THE UNIVERSITY
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Investigation of genetic and non-genetic factors influencing ewe reproductive performance in a Scottish hill flock

Ping Zhou

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The University of Edinburgh

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

Ping Zhou
Date: 16/12/2019

Abstract

Hill sheep farming is the backbone of the Scottish sheep farming industry. Due to the low productivity of hill sheep breeds and the changes in EU subsidy support system, the number of breeding ewes declined significantly between 2000 and 2018. This has affected the social, economic and environmental sustainability of rural areas. Introduction of lowland/upland prolific sheep breeds, such as the Lleyn, into hill farming systems might improve productivity of hill enterprises. However, the harsh hill weather conditions and poor nutritional supply in the hill environment might compromise the performance and the health of less adapted breeds, such as the Lleyn. The aim of this PhD project was to investigate the performance of Lleyn ewes farmed in a Scottish hill farm environment, comparing their performance with genetically unimproved Scottish Blackface (UBF) ewes and genetically improved Scottish Blackface (IBF) ewes farmed together in the same flock.

The study was conducted between November 2012 and October 2017 in two phases. In the first phase (between November 2012 and October 2015; the flock was comprised of approximately 300 ewes per genetic line), Lleyn ewes achieved significantly higher litter sizes at pregnancy scanning, lambing and weaning than UBF and IBF ewes, and had heavier average lamb birth weight than, and comparable average lamb weaning weight to, UBF and IBF ewes. In the second phase (between November 2015 and October 2017; the flock was comprised of approximately 200 ewes per genetic line), the three genetic lines of ewes were further challenged, with half of the flock being farmed under more extensive conditions. In this phase, based on the whole flock performance, Lleyn ewes achieved comparable litter sizes at pregnancy scanning, lambing and weaning to UBF and IBF ewes. They also achieved heavier average lamb birth weights and heavier weaned litter weights than UBF and IBF ewes.

Further investigations were performed to discover the possible reasons that led to performance differences among the three genetic lines. Firstly, Lleyn ewes were found to have higher pre-mating serum concentrations of vitamin D ($25(OH)D_3$ in particular) than UBF and IBF ewes, and these vitamin D parameters were positively associated with lamb birth weight in the following lambing season. Additionally, pre-

lambing metabolic profiles of twin-bearing ewes showed that they were well nourished in late pregnancy, with Lleyn twin-bearing ewes having higher magnesium concentrations than UBF and IBF ewes, which suggested that these Lleyns had higher feed intake. These outcomes could potentially assist Lleyn ewes to give birth to lambs with relatively heavier birth weight, which enhances lamb survival. The quality of colostrum secreted by Lleyn twin-bearing ewes, measured as Brix percentage, was as good as colostrum secreted by UBF and IBF twin-bearing ewes, and this is important for providing passive immunity and energy for neonates to survive. The post mortem examinations of the majority of dead lambs in three lambing seasons (2015-2017) showed that dystocia was the main cause of neonatal lamb death, with more IBF lambs dying as a consequence of this cause, compared to UBF and Lleyn lambs. Lleyn ewes had narrower external pelvic widths than UBF and IBF ewes, although no relationship between this measurement and lambing difficulty was found. Summer grazing behavioural observations showed that the number of ewes observed in different grazing sectors did not differ among the three genetic lines, suggesting no obvious differences in grazing behaviour. Overall, this thesis showed that Lleyn ewes have adapted well to the hill environment, and outperformed/equalled production levels of their UBF and IBF flockmates. Therefore, this lowland/upland sheep breed could be a good candidate for improving the productivity of hill sheep farming enterprises.

Lay summary

Hill sheep breeds have low productivity that leads to hill sheep farming enterprises being fragile and highly reliant on financial support. In the past 20 years, changes in subsidy payment systems resulted in significant reductions in the number of breeding ewes in Scotland. This downward trend has had negative impacts on the economy, community and environment in Scottish rural areas. Consequently, there is an urgent need to improve productivity of hill sheep farming systems in order to keep them viable. This PhD study investigated the performance of a possible breed substitute, a lowland/upland sheep breed. Lleyn ewes were farmed in a Scottish hill environment, and their performance was compared with that of a typical hill sheep breed – Scottish Blackface – those ewes comprising two different genetic lines (one line selected for average genetic merit across a number of ewe and lamb productions traits, UBF, and the other selected for high genetic merit, IBF).

The investigation was carried out across five consecutive production years. Between November 2012 and October 2015, 300 Lleyn ewes, co-grazing with 300 UBF and 300 IBF ewes, were introduced into hill conditions, although the flock was managed on semi-improved and improved pastures during key times in the production year, so was representative of Scottish hill farms with a less extensive management system. Under these conditions, Lleyn ewes had more lambs born and weaned per ewe mated than their UBF and IBF counterparts. The average birth weight of lambs born to Lleyn ewes was heavier than those of UBF and IBF flockmates, whereas the average lamb weaning weight did not differ among the three genetic lines. Ewes from all three genetic lines were then challenged further with half of the flock (balanced across genetic lines) being farmed under more extensive conditions for another two production years. During this period, based on the whole flock performance, the number of lambs born and the number of lambs weaned per ewe mated did not differ significantly among the three genetic lines. Lleyn ewes achieved heavier average lamb birth weight and heavier weaned litter weight. These traits are economically important for maintaining a financially sustainable hill sheep enterprise.

Several factors that might lead to ewe performance differences were investigated. The determination of ewe vitamin D status in serum samples collected in November 2015 showed that Lleyn ewes had higher vitamin D concentrations than UBF and IBF flockmates at that timepoint. These vitamin D parameters were positively associated with lamb birth weight in the following spring lambing season. The metabolic and mineral status of twin-bearing ewes in late pregnancy was gauged via assay of β-hydroxybutyrate, albumin, urea N, copper and magnesium plasma concentrations. The results were within the reference ranges for all three genetic lines, which indicated that twin-bearing ewes in the flock were well nourished. The higher magnesium concentrations found in Lleyn twin-bearing ewes suggested that they had higher feed intake compared to their UBF and IBF counterparts. During the lambing season, twin-bearing ewes' colostrum quality, which is important for lamb survival, was estimated using a refractometer and displayed as Brix percentage. The outcomes showed that the colostrum quality of Lleyn twin-bearing ewes was similar to that of colostrum from their UBF and IBF counterparts. The majority of dead lambs in 2015, 2016 and 2017 lambing seasons were examined post mortem, and dystocia (difficulties during birth) was found to be the main cause of neonatal lamb death, with almost double the number of IBF lambs dying due to this cause compared to UBF and Lleyn lambs. The average external pelvic width of Lleyn ewes was narrower than that of UBF and that of IBF ewes, although no association was found between this measurement and lambing difficulty. Additionally, there was no significant difference among the three genetic lines in the number of ewes observed grazing different sectors of the hill, during summer grazing behaviour observations.

This PhD project discovered that Lleyn ewes could give birth to lambs with relatively heavier birth weight, and weaned heavier litters compared to their UBF and IBF flockmates. This suggested that Lleyn ewes can adapt to a hill environment and perform well, and therefore, the adoption of this breed could be an option for improving the productivity of hill sheep farming systems.

Acknowledgements

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I wholeheartedly thank my supervisors, Dr Nicola Lambe (Scotland's Rural College), Dr Claire Morgan-Davies (Scotland's Rural College), Prof Neil Sargison (University of Edinburgh) and Dr Tom McEvoy (Scotland's Rural College), for their precious guidance and continuous support throughout my study. I would like to thank Prof Richard Mellanby for his support for the vitamin D analysis. I also gratefully acknowledge Dr Andrew Gill for his guidance and technical support during the vitamin D analysis in the Proteomics and Metabolomics laboratory at the Roslin Institute. Thanks to Dr Sarah Brocklehurst from BioSS, Dr Mark Hocart from Scotland's Rural College and Dr Liam Morrison from Roslin Institute for their guidance and support during my PhD study.

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Finally, thanks to my families, especially my husband for his unconditional support, and thanks to my daughter for her enormous moral support.

List of abbreviations

1α,25-$(OH)_2D$	1α,25-Dihydroxyvitamin D
1α,25-$(OH)_2D_2$	1α,25-Dihydroxyvitamin D_2
1α,25-$(OH)_2D_3$	1α,25-Dihydroxyvitamin D_3
24R,25$(OH)_2D_3$	24R,25-Dihydroxyvitamin D_3
25(OH)D	25-Hydroxyvitamin D
25(OH)D_2	25-Hydroxyvitamin D_2
25(OH)D_3	25-Hydroxyvitamin D_3
3-epi-25(OH)D	3-epi-25-Hydroxyvitamin D
3-epi-25(OH)D_2	3-epi-25-Hydroxyvitamin D_2
3-epi-25(OH)D_3	3-epi-25-Hydroxyvitamin D_3
ACTH	Adrenocorticotrophic hormone
BCS	Body condition score (referred to as CS in Chapter 5)
BF	Scottish Blackface
BMP15	Bone morphogenetic protein 15
BOHB	β-hydroxybutyrate
CON	Conventional Livestock Farming system
CRH	Corticotrophin releasing hormone
CV	Coefficient of variation
d	Day
DMEQ-TAD	4-[2-(3,4-Dihydro-6,7-dimethoxy-4-methyl-3-oxo-2-quinoxalinyl)ethyl]-3H-1,2,4-triazole-3,5(4H)-dione
EID	Electronic identification
FSH	Follicle-stimulating hormone
GDF9	Growth differentiation factor 9
GLM	Generalized Linear Model
GLMM	Generalized Linear Mixed Model
GnRH	Gonadotrophin-releasing hormone
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HPLC-MS/MS	High performance liquid chromatography tandem mass spectrometry
IBF	Improved Scottish Blackface

IGF1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IS	Internal standard
K_2HPO_4	Potassium phosphate dibasic
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
LFA	Less Favoured Area
LH	Luteinizing hormone
LLE	Liquid-liquid extraction
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LMM	Linear Mixed Model
M	Maintenance requirement
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
m/z	Mass/charge
Na_2HPO_4	Sodium phosphate dibasic
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PLF	Precision Livestock Farming system
se	Standard error
SD	Standard deviation
SG	Specific gravity
SPE	Solid phase extraction
SRUC	Scotland's Rural College
UBF	Unimproved Scottish Blackface
Urea N	Urea nitrogen

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Publications from the thesis

Paper

P Zhou, TG McEvoy, AC Gill, NR Lambe, CR Morgan-Davies, E Hurst, ND Sargison and RJ Mellanby. Investigation of relationship between vitamin D status and reproductive fitness in Scottish hill sheep. (Published on 04 February 2019 in Scientific Reports; [Sci Rep 9, 1162 (DOI https://doi.org/10.1038/s41598-018-37843-6)]

Abstracts

BSAS Annual Conference 2016 (Chester 2016)

A comparison of the reproductive performance of Lleyn and Scottish Blackface ewes mated to same-breed rams and managed together post-mating in a Scottish hill farm (Oral presentation; P Zhou, NR Lambe, CR Morgan-Davies, ND Sargison and TG McEvoy; Conference proceedings, Page 14)

67th EAAP Annual Conference (Belfast 2016)

Pre-lambing metabolic profile of twin-bearing ewes in a hill environment
(Poster presentation; P Zhou, NR Lambe, CR Morgan-Davies, ND Sargison and TG McEvoy; Book of abstract, Page 368)

9th International Sheep Veterinary Congress (Harrogate 2017)

Investigation of reproductive and health factors influencing breeding ewe fertility in hill flocks
(Oral presentation; P Zhou, N Sargison, N Lambe, C Morgan-Davies and T McEvoy; https://www.sheepvetsoc.org.uk/isvc2017)

Further experiments in relation to ewe reproductivity
(Poster presentation; P Zhou, N Sargison, N Lambe, C Morgan-Davies and T McEvoy; https://www.sheepvetsoc.org.uk/isvc2017)

Use of post mortem examinations in the investigation of perinatal lamb mortality (Poster presentation; P Zhou, N Sargison, N Lambe, C Morgan-Davies and T McEvoy; https://www.sheepvetsoc.org.uk/isvc2017)

Comparison of external pelvic conformation as a potential index of ease of lambing in different UK breeds and selected lines of ewes
(Poster presentation; P Zhou, N Sargison, N Lambe, C Morgan-Davies and T McEvoy; https://www.sheepvetsoc.org.uk/isvc2017)

BSAS Annual Conference 2018 (Dublin 2018)

Comparison of pre-lambing metabolic profiles of Scottish Blackface and Lleyn twin-bearing ewes farmed together in a Scottish hill environment
(Oral presentation; P Zhou, ND Sargison, NR Lambe, CR Morgan-Davies and TG McEvoy; Conference proceedings, Page 53)

Chapter 1: Introduction

Hill sheep farming is the backbone of the UK sheep industry, and it also plays an important role in rural economies and communities (SAC Rural Policy Centre, 2008; Thomson, 2011). Due to the low productivity of hill sheep breeds (Carson et al., 2001a) and the poor climatic conditions (compared to lowland/upland conditions) in the hill farming sectors, most of hill sheep farming enterprises are not financially viable without support (SAC Rural Policy Centre, 2008). With the combination of changes in the EU subsidy support system in the last 20 years (Morgan-Davies et al., 2015), the number of breeding ewes in Scotland have declined dramatically since 2000 (The Scottish Government, 2018a,b). The reduction of sheep grazing on hill lands has had negative impacts on biodiversity in those locations (SAC Rural Policy Centre, 2008; Pollock et al., 2013), with a decrease in farmland birds, rabbits and hares, but an increase in rank vegetation, deer, foxes, buzzards, corvids, goshawks (Thomson, 2011). Moreover, job opportunities in relation to farming and businesses that link between farming and other businesses have also been affected as a result of the reduction of breeding ewe population in Scottish farming system (SAC Rural Policy Centre, 2008). Therefore, improvement of productivity of hill sheep farming enterprises via key element, including genetic selection, farming breed substitutes and management of ewe nutrition should be investigated in order to maintain the sustainability of hill sheep farming system in Scotland.

1.1 Overview of the UK sheep industry

The UK sheep farming industry has been described as a three-tiered stratified pure- and cross-breeding system, in which many sheep are moved between different farms in hill and/or upland and/or lowland areas, in order to maximise their performance in terms of their inherent traits – hardiness of hill breeds, and prolificacy and meatiness of crossbreeds (Figure 1-1). The stratified system began in the 20th century, and it describes traditional sheep farming systems in the UK (Rodriguez-Ledesma et al., 2011).

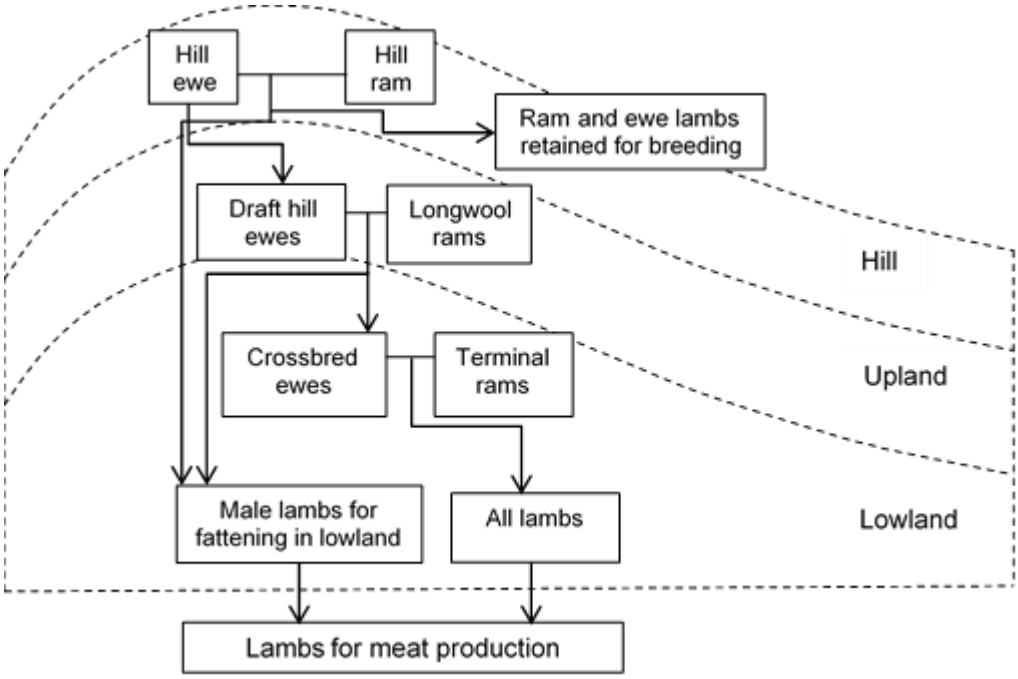


Figure 1-1. Description of the stratification of the UK sheep industry (Source: Rodriguez-Ledesma et al., 2011).

In this hill-upland-lowland system, hardy hill sheep breeds with lower prolificacy are being farmed in the hill sector, where hill ewes are mated with pure hill rams. Old hill ewes (~6 years old) are drafted to upland farms with milder conditions, where they are mated with long-wool crossing sires with characteristics of prolificacy and high growth rate, e.g. Bluefaced Leicester. Their female offspring are transferred to lowland farms where they are mated with Terminal Sires (e.g. Texel, Suffolk, Charollais) to produce finished lambs for meat production (Dwyer, 2008b; Rodriguez-Ledesma et al., 2011). However, this system poses a great risk of contagious disease transmission (Green et al., 2006). An example of disease outbreak due to sheep movement was the foot and mouth disease occurred in 2001. The cost of this epidemic was more than £8 billion, with millions of sheep culled for the purposes of either disease control or animal welfare (Anderson, 2002).

The stratified crossbreeding system dominated the UK sheep industry in the past, but its application has declined. The ratio of stratified to non-stratified crossbreeding systems decreased from 71:29 in 2003 to 55:45 in 2012 (Pollott, 2014). This change was caused by the combination of reduction in purebred ewe numbers, maintenance of crossbreeding in upland and lowland farming sectors, and overall declination of sheep numbers in the UK (Annett et al., 2011b; Pollott, 2014; Wolf et al., 2014;

Morris, 2017). A sheep survey conducted in 2012 showed that 'the wide range of *ad hoc* crossbreeding' is a new characteristic in UK sheep farming (Pollott, 2014). Within this 'new' system, the terminal sire breeds make a major genetic contribution in the sheep industry, and account for 68% of lamb production in the UK (Pollott, 2014).

1.2 Scottish sheep farming

The majority of Scotland (6.2 million hectares; approximately 80%) is used as agricultural land. Among these lands, more than 5.73 million hectares are classified as Less Favoured Area (LFA; Figure 1-2; The Scottish Government, 2018a), under European legislation (LFA – Article 2 of EU Council Directive No. 75/268/EEC). This classification means that these areas suffer from natural handicaps, such as poor climate, short growing seasons, mountainous or hilly topography, tendency towards depopulation, all of which constrain productivity and economic prosperity (Morgan-Davies et al., 2015). Over 62 percent of LFA has been used for sheep or mixed sheep and cattle farming (The Scottish Government, 2018b). This is an efficient way to convert poor quality forage into meat (protein) to meet the consumption requirement of the world's growing population. However, the low productivity of hill sheep breeds (Carson et al., 2001a) means that most hill sheep farming enterprises are not financially viable without support (SAC Rural Policy Centre, 2008). Additionally, changes in policy support over the years have had a negative effect on breeding ewes numbers in Scotland (Morgan-Davies et al., 2015). This trend started with the changes in the Common Agricultural Policy in 1992, which shifted support away from livestock production to more environmental protection and animal health and welfare support, with sheep numbers declining from a high of over 4 million. The numbers of breeding ewes declined almost continuously from 2000 to 2015, the exception being a slight increase in 2016 and 2017, and reached their lowest figure (2.56 million) since the beginning of the 21st century in 2018 (Figure 1-3; The Scottish Government, 2018a,b). The major reduction occurred in the North West of Scotland (SAC Rural Policy Centre, 2008) and most of the hill and upland areas in Scotland (Thomson, 2011), and that has had negative impacts on the economy, community and environment in those rural areas (SAC Rural Policy Centre, 2008; Thomson, 2011). The dependency on support needs to be altered to make the hill farming sector more sustainable. Therefore, solutions for improving reproductive

performance and overall productivity of hill sheep farming systems need to be investigated.

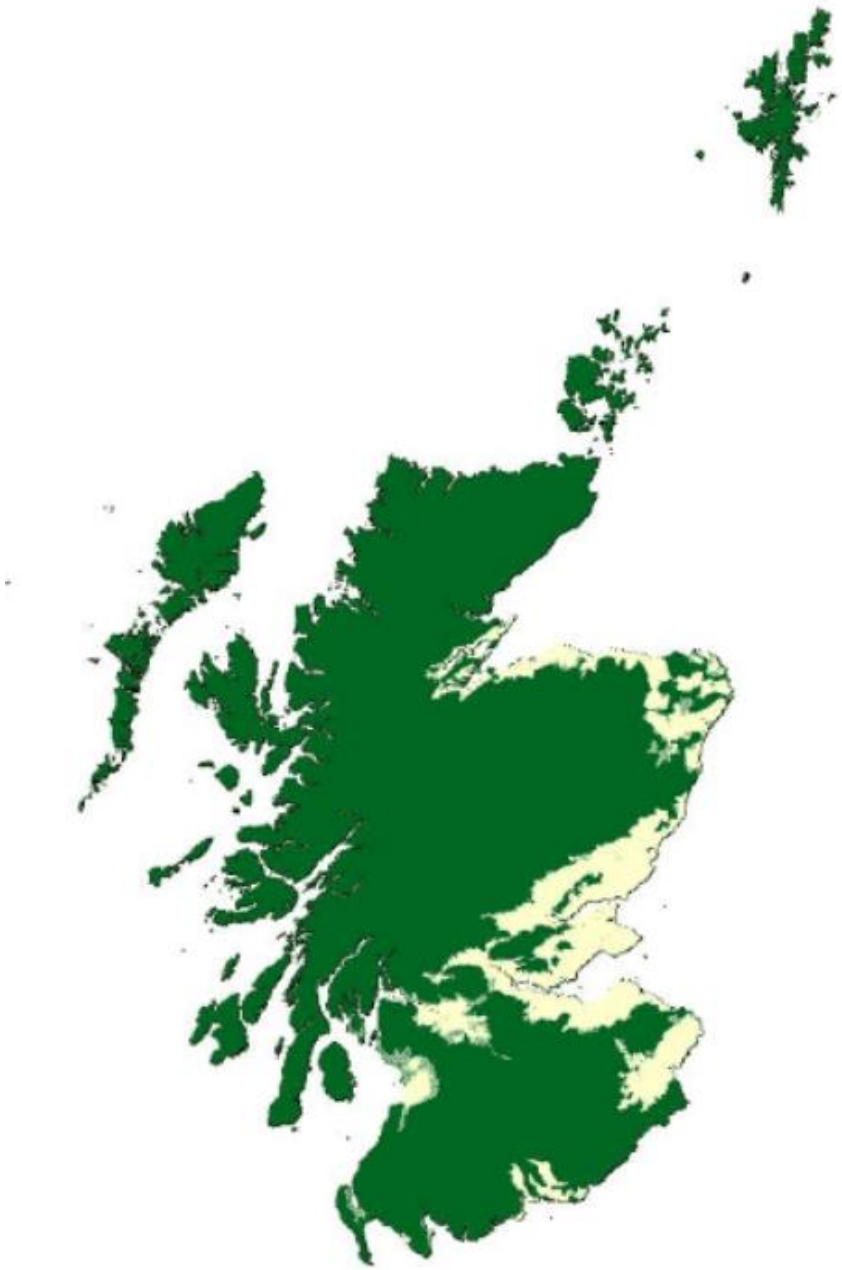


Figure 1-2. Less Favoured Areas map of Scotland. The dark green indicates the naturally disadvantaged land (LFA) in Scotland (Source: The Scottish Government, 2018a).

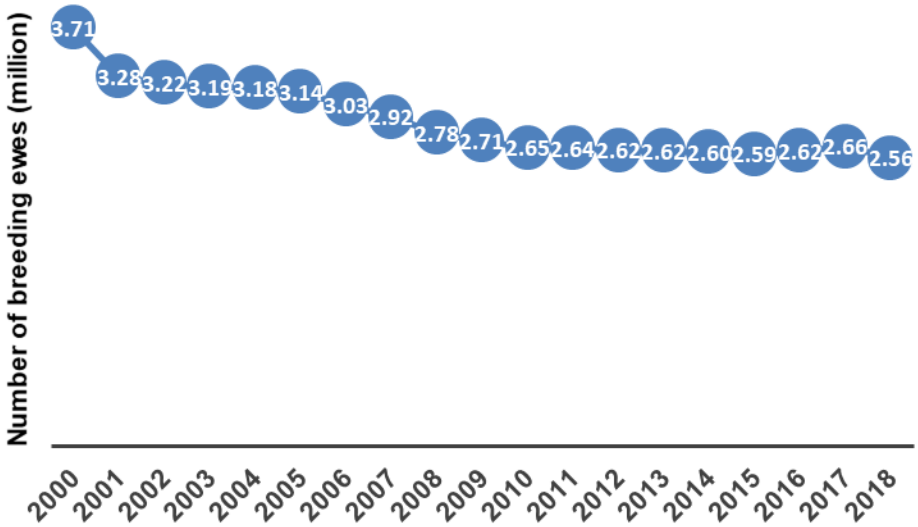


Figure 1-3. Number of breeding ewes in Scotland from 2000 to 2018 (Source: The Scottish Government, 2018a,b).

1.3 Hill farming conditions in Scotland

The majority of agricultural lands (over 3.6 million hectares) is rough grazing land due to the poor land quality (The Scottish Government, 2018b). Most of these lands are in the hills and uplands, which are composed of grassland, heath, mire, bracken and montane communities (Holland, 2001). The vegetation of the Scottish hills is largely semi-natural (Fenton, 1937), and the hills are commonly used for sheep extensive farming (Holland, 2001). The upland semi-natural grasslands are classified into two main categories. The 'good rough grassland' is on relatively dry lands with nutrient-rich soils, and is dominated by *Agrostis capillaris* and *Festuca ovina*; the 'poor rough grassland' is on wetter lands with more acidic soils, and is dominated by *Nardus stricta, Molinia caerulea* and/or *Juncus squarrosus* (Milne et al., 1998).

Scotland's climate is generally cool and very wet (Figure 1-4). The average annual temperature of Scotland between 2009 and 2018 was 7.7°C, and the average annual rainfall was 1585 mm for the same period (Kendon et al., 2019). The average annual sunshine duration was 1221 hours between 2009 and 2018 (Kendon et al., 2019).

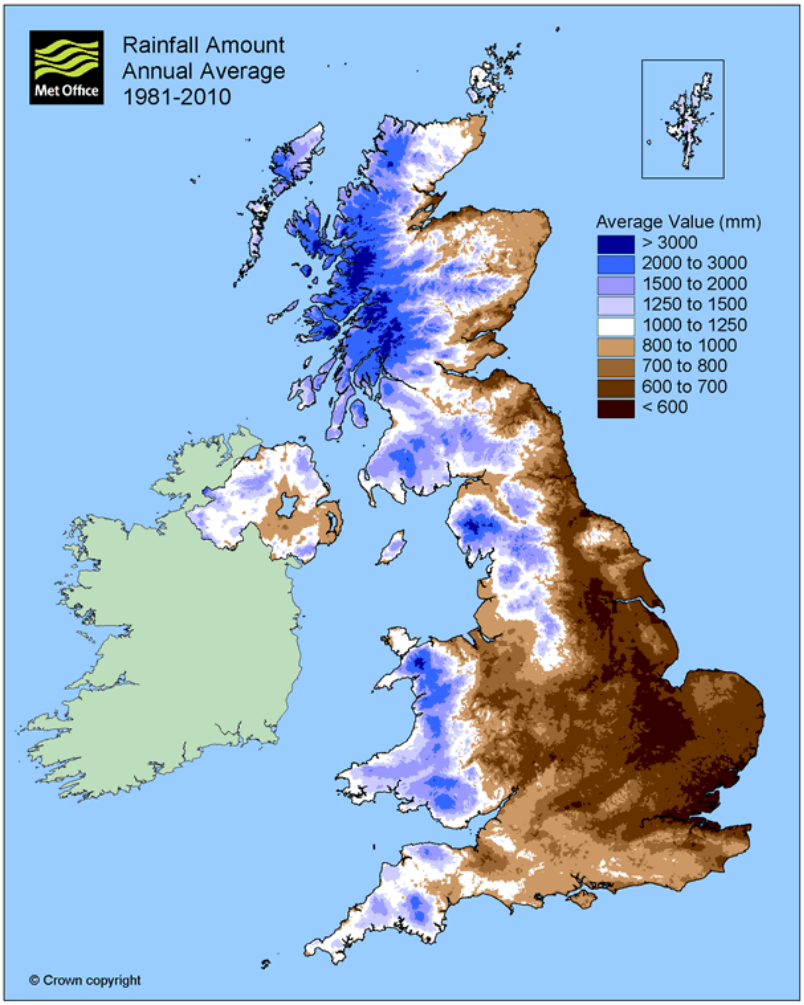


Figure 1-4. Average annual rainfall in the UK between 1981 and 2010 (Source: Met Office, 2017).

1.4 Sheep breeds in the UK

The sheep breeds farmed in the UK increased from 60 breeds in 1971 to 106 breeds in 2012. Such an increase is due to breeders importing foreign breeds, reimporting British breeds (e.g. Romney from New Zealand), and importing new composite breeds (e.g. Easycare; Pollott, 2014). Among the established breeds, the Scottish Blackface (BF) and the Lleyn are the most popular in the hill and the upland/lowland farming sectors, respectively (Pollott, 2014).

1.4.1 Scottish Blackface sheep

The three main breeds in the UK hill sector are the BF, Welsh Mountain and Swaledale (Pollott, 2014). Although the sheep breed structure has changed overtime, the BF remains the most popular breed being farmed in hill and mountain areas, with breeding ewe numbers of 1.68 million in 2003 and 1.12 million in 2012, and this breed is mostly kept for pure-breeding in the UK (Pollott, 2014). The characteristics of BF ewes are good ability to survive in harsh environments and good maternal traits (Dwyer & Lawrence, 1998, 2005). The bodyweight of mature ewes ranges between 45 and 70 kg depending on nutrition, environment and their strain (three strains: Perth, Lanark and Northumberland; National Sheep Association, 1998; The Blackface Sheep Breeders Association, 2020). Previous studies conducted in Northern Ireland hill farms reported that estimated mature weights of BF ewes were 52.8 and 53.8 kg (Carson et al., 2001a; Annett et al., 2011b). Purebred BF lambs have low birth weight, with an average of 3.5-4.5 kg for singletons, 3-4 kg for twins (National Sheep Association, 1998) and 3.5 kg for both singletons and multiple births (Morgan-Davies et al., 2008a). The prolificacy of BF ewes is 80-100% on harsh hills, 100-125% on better hills, or 125-180% on marginal ground (National Sheep Association, 1998).

1.4.2 Lleyn sheep

The Lleyn breed was derived from the Gwynedd breed, in North Wales, near the Lleyn Peninsula in the 18th Century (Yarwood & Evans, 2006). This lowland/upland medium-sized sheep breed is particularly prolific (up to 200% lambing rates in upland areas; Vipond et al., 2013) with easy lambing, so-called milkiness and good mothering traits in their typical farming environment (Houlden, 2013; Lleyn Sheep Society, 2018). Due to its characteristics, this breed has become increasingly popular. The number of breeding ewes increased from around 7,000 in 1971, to 237,000 in 2003, then 474,000 in 2012 (Pollott, 2014). The Lleyn has now become the most popular non-hill purebred breed in Britain (Pollott, 2014), and can be found in both lowland and upland farming systems (Ceyhan et al., 2015). The mature weight of ewes is up to 70 kg (National Sheep Association, 1998). The average birth weight of Lleyn lambs is 3.9 kg, which includes singletons and multiple births

(Ceyhan et al., 2015). Lleyn sheep from the Lleyn peninsula in Wales have been found to carry two major genes in which occurrence of specific point mutations has been found to be responsible for prolificacy (single copy) or infertility; these are *BMP15* (Bone morphogenetic protein 15*)* and *GDF9* (Growth differentiation factor 9; Mullen et al., 2013). Gene mutations could happen or are inherited, for example, at loci $FecX^{G}$ in the case of *BMP15* or $FecG^{H}$ in the case of *GDF9* or similar (Mullen et al., 2013), and when homozygous allelic mutations are present, infertility occurs in the female sheep (up to 26%; Vaughan et al., 1997).

1.5 Factors affecting ewe productivity

Ewe productivity in terms of lamb carcass production (number of carcass, carcass weight and carcass conformation) and wool production is the major contributor to flock profitability (Fogarty et al., 2003). Wool production can be affected by breed, maternal undernutrition, pregnancy status (Doney, 1964; Atkins, 1980; Ashworth et al., 2009), while a large number of factors can exert impacts on lamb carcass production. Figure 1-5 briefly illustrates the effects of key factors on ovulation rate (Griffiths et al., 1970; Doney et al., 1973, 1981a; Scaramuzzi & Radford, 1983; Gunn et al., 1984, 1991), embryonic mortality (Gunn et al., 1972; Gunn & Doney, 1975; Rhind et al., 1989; Robinson et al., 2002), placental development (Robinson et al., 2002; Redmer et al., 2004; Dwyer et al., 2005; Ashworth et al., 2011), foetal growth and development (Rhind, 2004; Redmer et al., 2004; Ashworth et al., 2005, 2011; Dwyer et al., 2005; Annett & Carson, 2006; Robinson et al., 2006; Gootwine et al., 2007; Muñoz et al., 2008, 2009; Sharma, 2010; Kenyon & Blair, 2014), colostrum production (Mellor & Murray, 1985; Gilbert et al., 1988; Dawson & Carson, 2002; Banchero et al., 2006; Higaki et al., 2013; Campion et al., 2019), milk production (Munro, 1962; Peart et al., 1975, 1979; Doney et al., 1981b,a, 1983; Gootwine & Pollott, 2000; Kenyon & Blair, 2014), maternal behaviour (Dwyer et al., 2003; Muñoz et al., 2009; Dwyer, 2014), neonatal behaviour (Slee & Springbett, 1986; Dwyer et al., 2001, 2003, 2005, 2010b,a, 2016; Muñoz et al., 2008) and lamb birth weight (Sidwell et al., 1964; Quirk & Norton, 1987; Fisher & MacPherson, 1991; Carson et al., 2001a; Dawson & Carson, 2002; Annett & Carson, 2006; Mitchell et al., 2007; Gardner et al., 2007; Speijers et al., 2010; Annett et al., 2011b; Kenyon & Blair, 2014; Elizalde et al., 2018), which would subsequently influence on carcass production. Additionally, lamb growth rate, that is an important parameter for flock

production (Ceyhan et al., 2015), can be affected by dam breed (Annett et al., 2011b), dam age (Dawson & Carson, 2002), sire breed (Elizalde et al., 2018), litter size and lamb sex (Sidwell et al., 1964; Kenyon & Blair, 2014), while carcass weight and quality can be affected by dam breed (Carson et al., 2001b), sire breed (Croston et al., 1987; Ellis et al., 1997), maternal nutrition (Greenwood & Thompson, 2007; Ashworth et al., 2009; Kenyon & Blair, 2014), nutrition for lamb (Carson et al., 2001b) and litter size (Kenyon & Blair, 2014). This PhD study focuses on how differences of ewe reproductive performance among different ewe breeds and ewe genotypes, along with consideration of the effects of ewe nutrition and harsh environmental conditions.

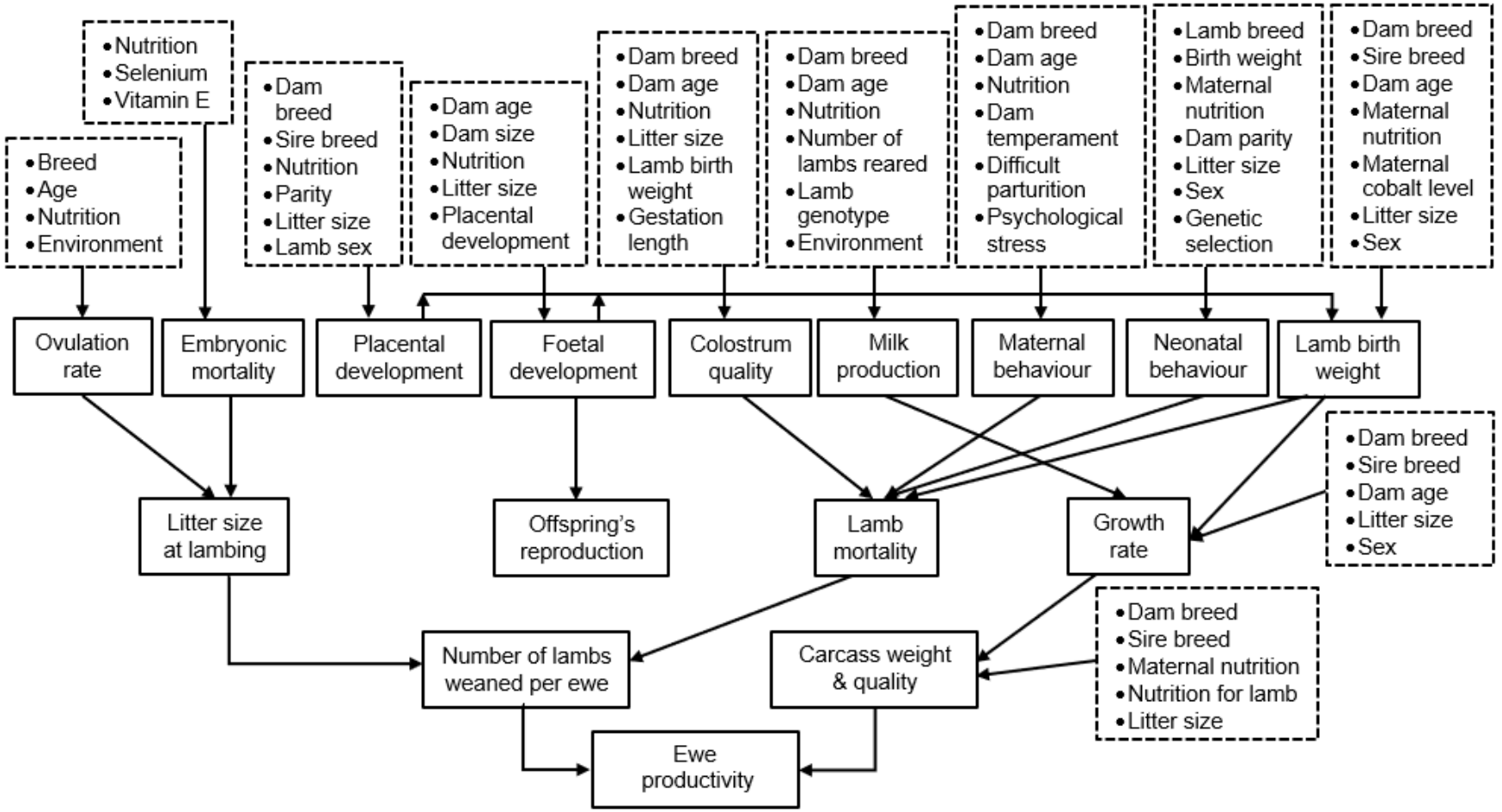


Figure 1-5. Lists of key factors that have effects on ewe productivity. The dashed boxes show the factors that have impacts on each ewe or lamb performance trait.

1.6 Genetic effects on ewe reproductive performance

Generally, extensive hill farming systems are dominated by purebred hardy hill sheep breeds with characteristics of low mature weight and producing light lambs at slaughter (Morris, 2017). Previous studies showed that farming crossbred ewes (Swaledale x BF, North Country Cheviot x BF, Lleyn x BF, Texel x BF) in hill conditions could improve flock performance as the results of increased litter size and lamb weight at weaning, compared with farming purebred BF ewes (Annett et al., 2011a). Nevertheless, application of crossbreeding in hill environment is very limited (Rodriguez-Ledesma et al., 2011) due to the constraints of managing the breeding programme on unfenced pastures.

An additional tool, genetic selection, has been applied in livestock farming for many decades (Simm et al., 1996) to improve flock performance progressively (Conington et al., 2001), as many traits associated with ewe and lamb performance have been found to be heritable. Signet (http://www.signetfbc.co.uk), the organisation delivering genetic evaluations of sheep and cattle to the UK industry, provides Estimated Breeding Values and Breeding Indexes to enable breeders to enhance the breeding potential of their ewes and rams (Signet Breeding Services, 2015). A study of selection indexes of hill sheep showed that multi-trait selection including improvement of maternal characteristics, along with the weight of lamb at weaning and at slaughter, was practicable, and that better productivity and profitability in the UK hill sheep enterprises can be achieved through genetic selection (Conington et al., 2001).

In the current Scottish farming industry, the breeding index applied for BF sheep is Hill 2 Index, which selects animals based on lamb body weight and growth rate, muscle and fatness, maternal ability, number of lambs born/reared and ewe body weight, whereas the breeding index applied for Lleyn sheep has been the Carcase+ Index, in which the focus is on selecting superior maternal ability, lamb growth rate, increasing the number of lambs reared (whilst avoiding large multiple litters), and enhancing carcase conformation with improved proportion of lean meat (Signet Breeding Services, 2014). A new Lleyn index has been offered through Signet since 2017, which focuses on selection of maternal ability of the ewe, lamb growth rate, increasing the number of successfully reared litters (whilst avoiding large multiple

litters), reducing ewe mature size, enhancing muscling and applying a penalty to animals with extreme leanness/fat breeding value (http://www.signetfbc.co.uk/sheepbreeder/latest-reports-sheep/lleyn/).

1.7 Non-genetic effects on ewe reproductive performance

Many other factors, including ewe age (Notter, 2000; Annett et al., 2011b), nutrition (Russel, 1991; Nottle et al., 1997) and environmental conditions (Dobson et al., 2012) can influence ewe reproductive performance, such as ovulation rate at mating (Nottle et al., 1997), embryonic mortality (Griffiths et al., 1970) and lamb birth weight (Wallace et al., 2011). In this section, the effects of nutrition and environmental conditions will be discussed briefly.

1.7.1 Regulatory mechanism influencing ewes' reproduction

The average length of an ovine (*Ovis aries*) oestrous cycle is 17 days (Edmondson et al., 2012). Female reproduction is regulated by the hypothalamic-pituitary-gonadal (HPG) axis with involvement of several hormones (Dobson et al., 2012; Joseph & Whirledge, 2017). The hypothalamus produces gonadotrophin-releasing hormone (GnRH) which influences the anterior pituitary gland, which in turn secretes follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both of which are transported via the bloodstream to the ovaries, where these two hormones promote the growth of follicles in the ovaries (Richards & Pangas, 2010; Edmondson et al., 2012). The ovary has an important physiological role in the reproduction process, being controlled indirectly by the hypothalamus in terms of ovulation and maintaining pregnancy. For example, during follicle growth, estradiol produced in the ovary provides positive feedback to the hypothalamus which, integrating all the information (physiological, seasonal and nutritional) determines whether zero, one or more eggs are ovulated in due course. The secretion of estradiol increases during follicular development (Figure 1-6). The level of estradiol reaches its peak when the follicles grow to mature size (average: 4-5 mm diameter; Downing & Scaramuzzi, 1991), which, in suitable circumstances, results in the LH surge, then ovulation occurs (Richards & Pangas, 2010; Edmondson et al., 2012).

After ovulation, the remaining cells of the follicle are differentiated (luteinised) and proliferate to form a corpus luteum (Richards & Pangas, 2010). Progesterone is produced by the corpus luteum, and this hormone provides negative feedback to the hypothalamus to reduce the secretion of GnRH, leading to a reduction in follicular development (Richards & Pangas, 2010). A higher level of progesterone also suppresses oestrus and ovulation. If a pregnancy is not established, the uterine endometrium will produce prostaglandin F2α, which causes death (luteolysis) of the corpus luteum, and a dramatic decline in progesterone secretion. The hypothalamus then begins to secrete more GnRH, and the follicular phase of the oestrous cycle culminates in oestrus and ovulation again (Liptrap, 1993; Edmondson et al., 2012; Figure 1-6).

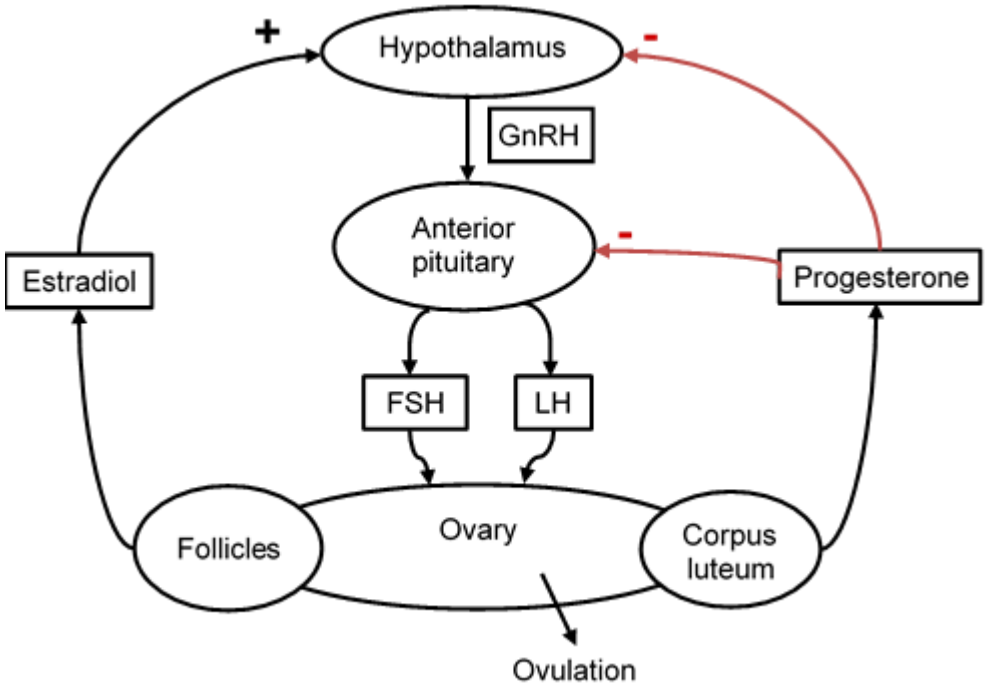


Figure 1-6. Endocrine control of the oestrous cycle in the ewe.

1.7.2 Stress effects on ewe reproduction

Stress is defined as any circumstance in which an animal cannot cope with its environmental or other conditions, and consequently cannot achieve its genetic potential for growth, reproduction and milk secretion (Dobson & Smith, 2000). There are many stressors, which include extreme environmental conditions, feeding

restrictions, diseases, altered metabolic demand and psychological challenges (Ralph et al., 2016). The neuroendocrine stress response is mediated by the animal's hypothalamic-pituitary-adrenal (HPA) axis. This complex physiological response is demonstrated in detail by Fink (2007), Romero et al. (2008) and Sheriff et al. (2011). Briefly, stressors (including physical and psychological) can activate the HPA axis resulting in increased concentrations of circulating glucocorticoids. In sheep, the principal glucocorticoid secreted in response to stress is cortisol. This steroid hormone can lead to negative reproductive outcome, by compromising function of the brain, the anterior pituitary gland and the ovaries (Ralph et al., 2016).

The HPA axis is inhibited by the hippocampus when an animal is in normal (non-stressful) conditions. When encountering challenging circumstances, the HPA axis is activated via stimulation the hypothalamus to secrete corticotrophin releasing hormone (CRH) and other secretagogues (e.g. arginine vasopressin), or its analogues (Sheriff et al., 2011; Joseph & Whirledge, 2017). These hormones promote the anterior pituitary to synthesise adrenocorticotrophic hormone (ACTH). After ACTH is released into the blood stream, it stimulates the adrenal cortex to secrete cortisol at a concentration above that for normal status (Figure 1-7; Sheriff et al., 2011). A measurable increase in cortisol status in vertebrates' blood via HPA axis normally requires 3 to 5 minutes (De Kloet et al., 2005).

Sheep farming systems require ewes to produce effectively in order to improve the production of the enterprises. Under severe stressful conditions, such as feeding restrictions and prevalence of diseases, animals will prioritise survival first, followed by growth, productivity and fertility (Qureshi, 2012). Reproduction will thus be firstly compromised when animals are under such circumstances (Oltenacu et al., 1980), via the influence of the HPA axis on the HPG axis leading to reproductive dysfunction in ewes (Narayan & Parisella, 2017). Chronic stress, such as food restriction, could be more likely to disrupt oestrous cycle and expression of oestrous behaviour in ewes, than acute stress (Wagenmaker et al., 2010). Previous studies revealed that increased stress-like cortisol concentration could reduce ewes' fertility by suppressing follicular maturation (Macfarlane et al., 2000), reducing GnRH pulse frequency in follicular phase (Oakley et al., 2009) and disrupting the preovulatory oestradiol rise and LH and FSH surges (Breen et al., 2005). Acute stress can inhibit the secretion of GnRH and gonadotrophin (Dobson et al., 2012).

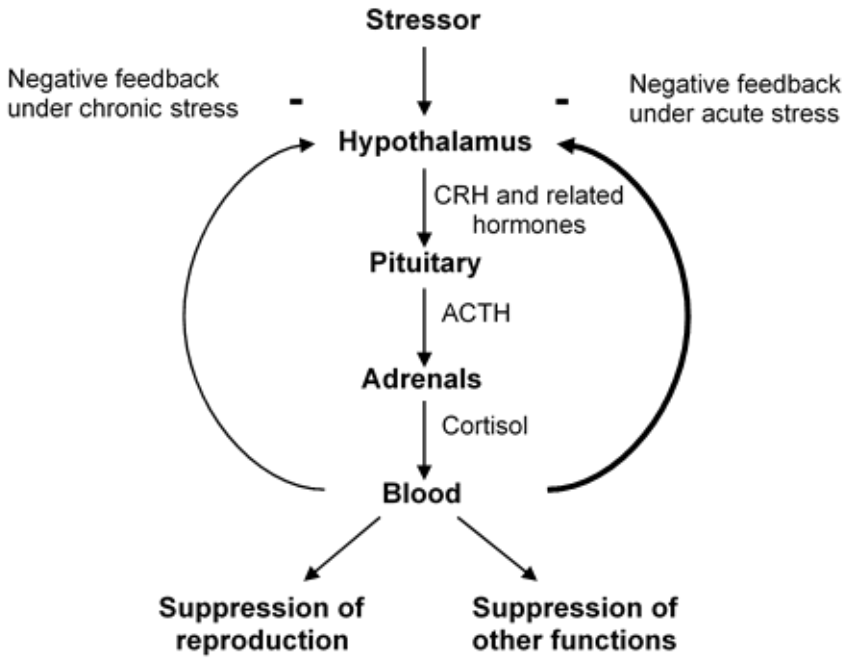


Figure 1-7. The hypothalamic-pituitary-adrenal (HPA) axis annotated to indicate negative feedback by cortisol and (below the axis) consequences of animal's response to stressors (Source: Sheriff et al., 2011).

During early pregnancy, ewes with undernutrition stress produce smaller, lighter and less well developed embryos (Parr et al., 1986), and increased embryonic mortality can be caused by a short period of severe nutrient restriction (Russel, 1991). Increased embryo loss was found among ewes with good body condition on a feeding regimen of 15% of maintenance requirement for seven days (Robinson, 1977). Research relating to day 2-16 post-mating nutritional treatments showed that embryo survival was not significantly affected by live weight (means for different subgroups: 41.7-50.0 kg) and body condition score (BCS; means for different subgroups: 2.48-2.93), but rather was influenced by feeding level with higher embryo survival rate being found among ewes fed with 100% of maintenance requirement than among those fed with either 25% or 200% of maintenance requirement (1.19 *vs.* 1.12 or 1.04, respectively; Cumming et al., 1975).

Moreover, placenta is the organ that transports respiratory gases, nutrients and wastes between maternal and foetal systems. The development of placenta is essential to ensure adequate transplacental exchange, and so to support foetal growth (Reynolds et al., 2005). The number of cotyledons formed and their

subsequent size and vascularity can be influenced by maternal nutritional status (Cross & Mickelson, 2008). Previous studies reported that undernutrition in pregnant ewes could result in reductions in the size of cotyledon and uterine blood flow (Chandler et al., 1985; Kelly et al., 1992). However, the effects of undernutrition during pregnancy in adult ewes are not consistent, as other studies found no effects on uterine and umbilical blood flows (Leury et al., 1990; Newnham et al., 1991). Additionally, nutrition restriction has influence on foetal development. A study showed that ewes fed with 50% of National Research Council requirement from days 28 to 78 of gestation had smaller and lighter foetuses, with lighter liver, lung and kidney, and shorter crown rump length, compared to those fed with 100% of National Research Council requirement (Vonnahme et al., 2003).

On the other hand, ewes may be tolerant to some stressful conditions, such as poor forage quality. McEvoy & Robinson (2002) reviewed experiments on the effects of undernutrition on sheep reproductive performance, and found that mild undernutrition during mid-pregnancy in adult ewes with good BCS (3.5), but not those with poor BCS (≤2.0) or in young growing ewes, had no effects on placental and foetal growth. This indicates that adult pregnant ewes with good body condition score can mobilise their body reserves to maintain a balanced nutrient supply to their foetuses during chronic undernutrition. Another study showed that foetuses carried by Border-Leicester x Merino ewes fed to meet 70% of energy requirements from day eight of gestation to term had more perirenal adipose tissue (an essential fuel for maintaining body temperature in neonates; Symonds & Lomax, 1992) than their counterparts carried by ewes fed to meet 100% of energy requirements (Budge et al., 2004). Therefore, stress may not always impair, and sometimes it may enhance, ewe reproductive performance. How animals respond to undernutrition (a stressor) is also associated with their breed (tolerance of unfavourable environmental conditions), age, maturity, long-term nutrition (i.e. ewe BCS), phase of gestation and the severity of undernutrition (McEvoy & Robinson, 2002; Narayan & Parisella, 2017).

1.7.3 Nutrition and ewe reproduction

As a stressor, the effects of undernutrition on ewe reproductive performance are complex, and have been substantially studied and reviewed (Cumming et al., 1975; Russel, 1991; Nottle et al., 1997; Robinson et al., 1999, 2002, 2006; McEvoy & Robinson, 2002; Ashworth et al., 2005, 2009; Annett & Carson, 2006; Muñoz et al., 2008). Maternal nutrition not only provides essential nutrients (including glucose, amino acids, vitamins and minerals) for foetal development, but also can exert effects on endocrine mechanisms to modify the secretion of hormones, which influences oocyte development, ovulation rate, embryo survival, placental development, foetal growth and lamb vigour (Robinson et al., 1999, 2002). Thus management strategies in relation to maternal nutrition are important when aiming to make the most of ewe genetic potential for reproduction.

1.7.3.1 Measurements of nutritional status

Body condition scoring is a subjective measurement of the proportion of fat and muscle in a live sheep, that is not affected by the size of the animal or gut-fill (Russel et al., 1969). This method has been widely accepted as an on-farm tool to reflect past nutritional status of sheep, and helps decision-making in terms of flock nutrition and management (Phythian et al., 2012). It also has been suggested as a tool in relation to flock health planning programmes (Sargison & Scott, 2010) and animal welfare assessment (Morgan-Davies et al., 2008b). In extensive farming systems, both changes in ewe body weight and BCS can be used to estimate the degree of under-nutrition of ewes in early and mid-pregnancy (Russel, 1984, 1991). In late pregnancy, due to the rapid growth of the foetus, examination of concentrations of circulating metabolites is a preferred means for determining ewe nutritional status (Russel, 1991; Caldeira et al., 2007).

1.7.3.2 Nutritional effects on ovulation rate

Ewe ovulation rate is an important determinant for the number of lambs weaned per ewe in a sheep farming system (Knight, 1990). This reproductive trait is highly

dependent on long-term nutritional supply. Follicles require approximately six months to develop from the primordial stage (Robinson et al., 2002). During development, specific nutrients, such as amino acids and volatile fatty acids, are required for follicle growth (Robinson et al., 2002). Although follicular atresia (a normal degenerative process; Hsueh et al., 1994) occurs at various rates in each developmental stage, improved feeding regimens can reduce its magnitude at critical stages, and lead to increased ovulation rate (Robinson et al., 2002). Body weight and BCS at mating reflect long-term nutritional status of ewes. The former parameter has a curvilinear relationship instead of linear relationship with ovulation rate, thus additional weight gain over the optimum would not result in an increased ovulation rate (Smith, 1991). Previous studies showed that BCS of BF ewes at mating was positively associated with ovulation rate, when ewe BCS was between 1 and 3.5 (Gunn et al., 1969; Gunn & Doney, 1975). In adult ewes, there are three critical time points when ovulation rate is particularly sensitive to nutrition. Firstly, six months before mating when ovarian follicles leave the primordial pool and become committed to growth. Undernutrition at that stage reduces the number of follicles leaving the primordial pool and becoming available for ovulation. Additionally, short-term improvement of ewe nutrition by flushing during the 10 days prior to mating, or thirdly just between day eight to day four prior to mating, can improve ovarian follicular growth and oocyte quality, and reduce follicular atresia, and thus improve ovulation rate (Robinson et al., 2002, 2006). A notable experiment, indicative of the short-term influence of nutrition, is one which showed that Merino ewes on 8 weeks of low feeding regimen (inducing 10-20% ewe body weight loss) started 6 months before ovulation that had received lupin supplement 10 days before ovulation had significantly higher ovulation rates than their counterparts that had not received lupin supplement (1.63 *vs.* 1.06; Nottle et al., 1997). In contrast, another experiment conducted by the same authors showed that Merino ewes on 8 weeks of high feeding regimen (maintaining ewe body weight) started 4 months before ovulation that fed with lupin supplement 10 days before ovulation did not improve ovulation rates, compared to their counterparts that had not received lupin treatment (1.67 *vs.* 1.64; (Nottle et al., 1997). This suggests that ewes with good body conditions do not response to flushing, as their long-term and current nutrition has minimized follicle atresia and maximised ovulation rates (Robinson et al., 2002).

Nutritional effects on ovulation rate are mediated by reproductive and metabolic hormones (Scaramuzzi et al., 2006). Offering ewes a diet of hay with supplementary feeding of grain can increase the weight of pituitary gland and so increase total LH and FSH concentrations available to stimulate the growth of follicles (Edmondson et al., 2012). Additionally, Cheviot ewes on (in relative terms) a high feeding regimen (0.8 kg dry matter/head/day) had a higher LH pulse frequency and higher prolactin concentration in the follicular phase than those on a low feeding regimen (0.3 kg dry matter/head/day; Rhind et al., 1985). Moreover, ewes with relatively high BCS (BF, mean BCS: 2.86) were found to have higher FSH and prolactin status in the entire subsequent oestrous cycle than ewes with low BCS (mean BCS: 1.84), and that resulted in a higher ovulation rate and larger follicles (≥ 4 mm in diameter) in the higher BCS ewes (Rhind & McNeilly, 1986). Lastly, for now, a study of BF ewes showed that subclinical deficiency of cobalt could result in reduced ovulatory response (Mitchell et al., 2007). Therefore, improvement of nutritional supply prior to and during mating can enhance ewe reproductive performance and flock productivity.

1.7.3.3 Maternal undernutrition and lamb birth weight

The review of some previous publications relating to the effects of maternal undernutrition on lamb birth weight are summarised in Table1-1. Undernutrition during early- and mid-pregnancy is less likely to exert effects on lamb birth weight (Parr et al., 1986; Kleemann et al., 1993; Rae et al., 2002a; Gopalakrishnan et al., 2004; Annett & Carson, 2006; Gardner et al., 2007; Debus et al., 2012; Kenyon & Blair, 2014). The retardation of foetal growth led by maternal nutrition restriction during early- and mid- pregnancy can be alleviated, if nutritional supply is adequate during late pregnancy (Greenwood & Thompson, 2007). Many experiments revealed that maternal undernutrition in late pregnancy can lead to a reduction in lamb birth weight (Taplin & Everitt, 1964; Borwick et al., 2003; Wu et al., 2006; Gardner et al., 2007; Tygesen et al., 2007; Kenyon & Blair, 2014). However, the study conducted by Deligeorgis et al. (1996) showed that ewes fed with 90% of the maintenance requirement during mid- and late pregnancy had no effect on lamb birth weight, compared to those fed with 110% of the maintenance requirement. Moreover, lambs born to younger primiparous dams often had lighter birth weight than those born to

older multiparous ewes (Annett & Carson, 2006; Gardner et al., 2007; Loureiro et al., 2011) despite there being other findings suggesting that no effect of dam age and parity on lamb birth weight (Trail & Sacker, 1969; Macedo & Hummel, 2006). These different outcomes could be due to the differences in the dam age and the body condition at her first pregnancy, as ewe body weight and BCS both have influence on lamb birth weight (Kenyon et al., 2004). Additionally, different breeds might have different responses to maternal undernutrition. Rooke et al. (2010) reported that maternal nutrition restriction (to 75% instead of 100% of the energy requirement) from day 1 to day 90 of gestation significantly reduced birth weight of Suffolk lambs, but not of BF lambs. Therefore, the effects of maternal undernutrition on lamb birth weight are depend on breed, age, stage of pregnancy, ewe body condition, severity and length of undernutrition, and other factors, such as environmental conditions.

Lamb birth weight is an important factor for lamb survival. Firstly, light lambs have less brown adipose tissue (1-2% of lamb birth weight) compared to heavy lambs (Symonds & Lomax, 1992). Brown adipose tissue can rapidly provide large amounts of heat via activation of mitochondrial uncoupling protein, which allows free-flow of protons across the inner mitochondrial membrane, resulting in rapid dissipation of chemical energy as heat rather than as adenosine triphosphate (Cannon & Nedergaard, 1985, 2004). For neonatal lambs, heat is generated through non-shivering thermogenesis during metabolism of brown adipose tissue (Symonds & Lomax, 1992), that is critical for lamb survival in the first 24 hours after birth (Robinson, 1981). Moreover, light lamb birth weight has subsequent effects in terms of reduced lamb vigour and performance (Slee & Springbett, 1986; Dwyer et al., 2016), and that can lead to higher mortality rate (Rooke et al., 2015).

Table 1-1. The effect of maternal feeding regimen and the length and the timing of feeding treatment on lamb birth weight.

Reference	Timing and length of nutritional treatment	Nutritional treatment	Lamb birth weight
Annett & Carson. (2006)	Day 1 of gestation (d1) to d31	0.6 M (maintenance requirement) *vs.* 1.0 M *vs.* 2.0	No effect on birth weight
Borwick et al. (2003)	d100 to parturition	0.7 M *vs.* 1.0 M	0.7 M lighter at birth weight
Corner et al. (2008)	d70 to d107 to d147	2-2, 2-4, 4-2, 4-4 cm swards	2-2 and 4-2 lighter birth weight than 4-4
Debus et al. (2012)	15 days pre-mating (d-15) to d30	0.5 M *vs.* 1.0 M	No effect on birth weight
Deligeorgis et al. (1996)	d30 to parturition	0.9 M *vs.* 1.1 M	No effect on birth weight
Gardner et al. (2007)	d1 to d30, d31 to d80, d110 to d147	Different models	Only d110 to d147 nutrition treatment affected birth weight
Gopalakrishnan et al. (2004)	d0 to d95	0.5 M *vs.* 1.0 M	No effect on birth weight
Gunn et al. (1995)	d47 to parturition	Low *vs.* high	Undernutrition reduced birth weight
Kleemann et al. (1993)	d50 to d100	Low *vs.* high	No effect on birth weight
Kotsampasi et al. (2009)	d0 to d30 and d31 to d100	0.5 M *vs.* 1.0 M	No effect on birth weight
Morris & Kenyon (2004)	d64 to parturition	2, 4, 6, 8 cm swards	2 cm lighter at birth weight
Muñoz et al. (2009)	d0 to d39 and d40 to d90	0.6 M *vs.* 1.0 M *vs.* 2.0 M and 0.8 M *vs.* 1.4	0.6 M lighter at birth weight
Nordby et al. (1987)	d-30 to d100	0.7 M *vs.* 1.1 M	0.7 M lighter at birth weight
Oliver et al. (2005)	d-61 to d30	Loss of 15% weight or well fed	Lost weight lighter at birth weight
Parr et al. (1986)	d1 to d35	0.5 M *vs.* 1.5 M	No effect on birth weight
Rae et al. (2002a)	d1 to d95	0.5 M *vs.* 1.0 M	No effect on birth weight
Rooke et al. (2010)	d1 to d90	0.75 M *vs.* 1.0 M	BF: no effect on birth weight Suffolk: 0.75 M lighter at birth weight
Smith et al. (2010)	d-28 to d7	0.7 M *vs.* 1.1 M	No effect on birth weight
Taplin & Everitt (1964)	d0 to d90 and d91 to parturition	Loss or gain 25% body weight	Undernutrition in late pregnancy reduced birth weight
Tygesen et al. (2007)	Last 6 weeks of gestation	0.6 M *vs.* adequate	0.6 M lighter at birth weight

1.7.3.4 Maternal undernutrition and offspring reproductive performance

Sheep adult reproductive performance can be affected by maternal nutrition during pregnancy. Previous publications have demonstrated that keeping ewes on restricted feeding regimens at different stages during pregnancy can result in reduced folliculogenesis in foetal ovaries (Rhind, 2004; Ashworth et al., 2005). Arndt et al. (2006) observed that maternal restriction (60% of maintenance) from day 50 to day 135 of gestation reduced cellular proliferation in foetal ovarian primordial follicles. Maternal undernutrition also has adverse effects on the development of the foetal ovary before gonadotrophin secretion from the foetal pituitary begins (day 65 of gestation), and before gonadotrophin receptors are present on the foetal ovaries (after day 135 of gestation; McEvoy & Robinson, 2002). Rae et al. (2002a) investigated the effects of maternal undernutrition on their offsprings' reproductive performance and found that female offspring born to under-nourished ewes (BF; 50% of maintenance during the first three months of gestation) had lower average ovulation rate at 20 months old (1.17 *vs.* 1.46) than their counterparts born to well-nourished ewes (100% of maintenance). This level of maternal undernutrition had no effect on foetal pituitary function (Rae et al., 2002b). Another study showed that feeding BF ewes 70% of energy requirements from day 100 of gestation to term did not affect the onset of puberty and the hypothalamic-pituitary function of their offspring, compared to offspring of ewes fed 100% of energy requirements (Borwick et al., 2003). Therefore, maternal undernutrition could have a direct impact on foetal ovarian development (Robinson et al., 2006), even if undernutrition is imposed during the first 30 days, subsequent 20 days or between day 50 and day 65 of gestation (Ashworth et al., 2005). Such effects could mean a lifetime suppression of ovulation rate in ewes (Gunn et al., 1995). Maternal undernutrition can result in foetal hypoglycaemia. Glucose in the ovine foetus has a role in regulation of circulating insulin-like growth factor 1 (IGF1), with IGF1 status being influential in activating cell proliferation and differentiation of reproductive organs via autocrine and/or paracrine mechanisms (Nayak & Giudice, 2003; Tian et al., 2004). An experiment showed that female offspring born to ewes fed to meet 50% of energy requirements from ~ day 100 of gestation to term had lighter uterus, lower levels of ovarian and uterine mRNA expressions of IGF1 and its receptor, smaller follicles, and lower numbers of endometrial glands at two months old, compared to their counterparts born to ewes fed to meet 100% of energy requirements (Hoffman et al.,

2018). This indicates that maternal undernutrition could impair reproductive tract and organ development in the offspring's early life.

1.7.3.5 Maternal undernutrition and ewe-lamb bond

After lambing, licking and grooming provided by ewes are important for establishing olfactory memory in ewes and facilitating bonding between the ewe and her lambs (Dwyer et al., 2016). This also encourages the lamb to suck and survive. One study revealed that underfeeding ewes (offered 65% of the feeding regimen provided to a high intake treatment group) from week four of gestation to full term had negative impacts on the time spent licking the lamb, and the development of the bond between ewe and lamb, compared to ewes fed with the high intake feeding regimen (energy intake (MJ metabolisable energy/day) according to week 4 of gestation to lambing: 5.2-12.9 for single-bearing ewes, 5.2-15.8 for twin-bearing ewes, 5.2-16.8 for triplet-beairng ewes; Dwyer et al., 2003). Muñoz et al. (2008) reported that lambs born from ewes fed a mild undernutrition regimen (80% of predicted metabolisable energy requirements for maintenance) in mid-pregnancy could have improved neonatal behaviour, as indicated by standing up and attempting to suck sooner than counterparts born from ewes fed a high feeding regimen instead (140% of predicted metabolisable energy requirements for maintenance). A statistically significant interaction between maternal undernutrition and parity has been reported in relation to ewe-lamb bonding. Undernutrition during early and mid-pregnancy impairs acceptance of lambs by primiparous dams (Muñoz et al., 2009), but not in the case of multiparous dams (Muñoz et al., 2008). The effect of maternal feeding restriction on neonatal lamb behaviour also varies between breeds. Lambs of Suffolk ewes that had been undernourished from day 1 to day 90 of gestation but not lambs from correspondingly undernourished BF ewes, took longer to stand and suck than those born from well-nourished ewes (Dwyer et al., 2010a,b).

1.7.4 Vitamin D status and ewe reproductive performance

Previous studies in humans reported that maternal vitamin D deficiency had negative impacts on reproduction, with increased risk of pregnancy loss (Mumford et al., 2018) or having low birth weight (Wang et al., 2018). A recent study of Soay

sheep in Scottish island, St Kilda, found that ewe vitamin D concentration was positively associated with number of lambs reared to one year old (Handel et al., 2016), that is an important trait for flock productivity. Therefore, the metabolism of this vitamin and their effects on reproductive performance are reviewed briefly in this section.

1.7.4.1 Sources of vitamin D

Vitamin D is a fat-soluble steroid hormone. It has two principal forms – ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Both vitamin D_2 and D_3 contents are extremely low in plant resources (Combs, 2012). However, with sufficient exposure to ultraviolet B radiation (290-315 nm; Holick, 2007), humans and other vertebrates can convert 7-Dehydrocholesterol (provitamin D_3) in the skin into previtamin D_3 and then vitamin D_3 (Combs, 2012; Figure 1-8). After consumption, or synthesis in the skin, the two forms of vitamin D undergo similar complex metabolic processes. Firstly, vitamin D (including vitamin D_2 and D_3) binds to vitamin D binding protein on entering into systemic circulation, and it is transported to the liver, where vitamin D is hydroxylated to the main circulating forms, 25-Hydroxyvitamin D_2 ($25(OH)D_2$) and 25-Hydroxyvitamin D_3 ($25(OH)D_3$), respectively. This conversion is catalysed by 25-Hydroxylases (CYP2R1, CYP27A1, CYP3A4 and CYP2J3). 25-Hydroxyvitamin D (25(OH)D, consisting of $25(OH)D_2$ and $25(OH)D_3$) is then transported to the kidney, where 25(OH)D is further hydroxylated to 1α,25-Dihydroxyvitamin D ($1\alpha,25\text{-}(OH)_2D$, consisting of 1α,25-Dihydroxyvitamin D_2 ($1\alpha,25\text{-}(OH)_2D_2$) and 1α,25-Dihydroxyvitamin D_3 ($1\alpha,25\text{-}(OH)_2D_3$)), the biologically active form of vitamin D, by 1α-Hydroxylase (CYP27B1). One of the main circulating vitamin D metabolites, $25(OH)D_3$, can be deactivated into 24R,25-Dihydroxyvitamin D_3 ($24R,25(OH)_2D_3$) via side-chain oxidation catalysed by 24-Hydroxylase (CYP24A1; Prosser & Jones, 2004; Jones, 2012; Kasalová et al., 2015; Bikle, 2018). C3-epimerisation is a second biochemical pathway, by which 25(OH)D can be converted to a corresponding epimeric form, 3-epi-25-Hydroxyvitamin D (3-epi-25(OH)D; Singh et al., 2006).

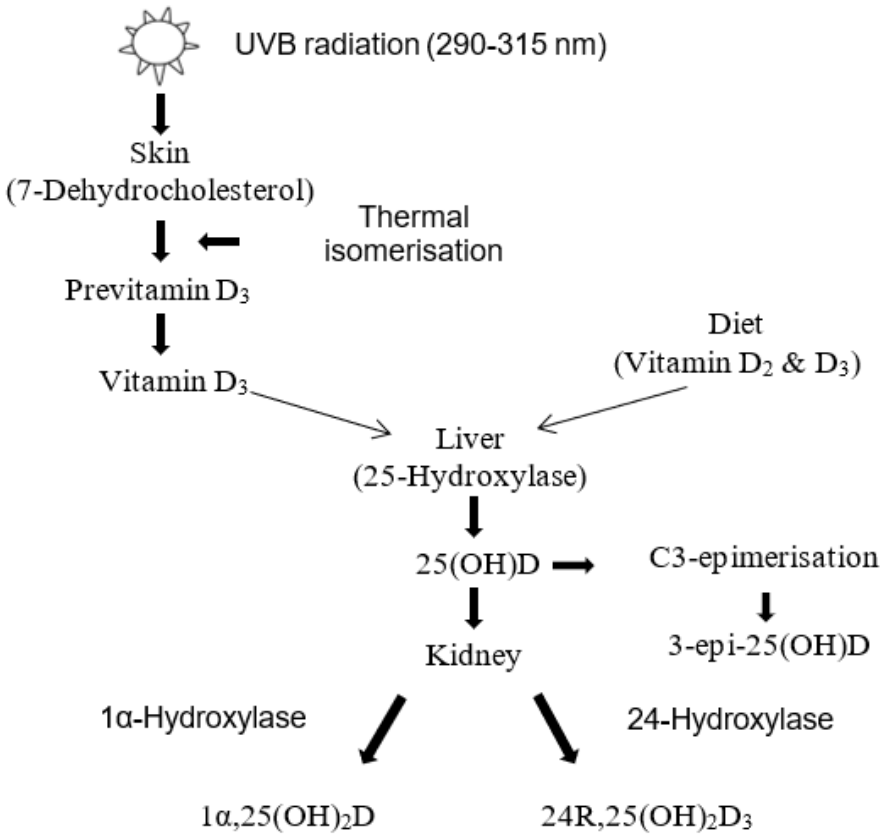


Figure 1-8. Vitamin D metabolism.

1.7.4.2 Vitamin D deficiency and associated factors

Vitamin D status has been intensively investigated in recent years. Several studies showed that vitamin D insufficiency in humans is a common issue worldwide, with approximately one-third of the United States population and approximately 1 billion people throughout the world having vitamin D deficiency (serum 25(OH)D<50 nmol/l; Holick 2007; Looker et al. 2011; van Schoor & Lips 2011). Vitamin D deficiency is not only a cause of bone disease, but also is a risk factor for autoimmune disease, cardiovascular disease, cancer, infection and reduced reproductive performance (Tsiaras & Weinstock, 2011; Combs, 2012). The effect of vitamin D concentration on animal health and performance has also been studied (Kinuta et al., 2000; Coffey et al., 2012; Kohler et al., 2013). Nemeth et al. (2017) noted three vitamin D status ranges that suit most species, including sheep. These

are as follows: vitamin D deficiency: serum 25(OH)D<25 nmol/l; vitamin D insufficient: 25-75 nmol/l; vitamin D sufficient: 75-150 nmol/l.

Vitamin D status of each individual can be affected by many factors, such as latitude, altitude, season, duration of sunshine exposure, as well as by age and skin pigmentation (Need et al., 1993; Engelsen et al., 2005; Tsiaras & Weinstock, 2011; Kohler et al., 2013; Willems et al., 2013). In hair-coated animals, the colour and the thickness of the coat or fleece have effects on vitamin D_3 photobiosynthesis (Horst & Littledike, 1982; Mearns et al., 2008; Dittmer & Thompson, 2011; Handel et al., 2016). Conversely, Hymøller & Jensen (2010) reported that vitamin D_3 was evenly synthesized throughout the whole body surface in hair-coated Danish Holstein dairy cows, irrespective of the amount of hair coverage on the site.

1.7.4.3 Vitamin D and reproductive performance

Vitamin D receptors and vitamin D metabolising enzymes, including CYP2R1 & CYP27A1 & CYP27B1, are present in many reproductive tissues, such as ovaries, uterus, testes and the epididymides (Bikle, 2009; Jensen, 2014; Brozyna et al., 2015; Yao et al., 2018). Therefore, it is hypothesized that vitamin D deficiency can impair reproductive performance.

The presence of vitamin D receptor and vitamin D metabolising enzymes in the gonads is important for regulating the status of intracellular 1α,25$(OH)_2D_3$ and the activation of vitamin D receptor, and that supports successful reproductive performance (Boisen et al., 2016). One experiment showed that, in pregnant women, the expressions of CYP27B1 in the placenta and decidua were higher in the first and second trimesters than in the third trimester (Zehnder et al., 2002). The authors suggested that this vitamin D metabolising enzyme had a role for implantation and/or placentation (Zehnder et al., 2002). In males, vitamin D receptor expression and vitamin D inactivating enzyme CYP24A1 expression have been found to be positively associated with semen quality, and vitamin D has impacts on sex steroid production and oestrogen signalling (Boisen et al., 2016). An experiment on rams (Hu sheep) at different developmental stages suggested that vitamin D receptor and vitamin D metabolizing enzymes had a potential influence on

spermatogenesis, as the expression levels of vitamin D receptor and vitamin D metabolizing enzymes in the male reproductive tract (i.e. testes and cauda) increased with age (3-, 9- and 24-month old rams; Yao et al., 2018). The expression levels of vitamin D receptor and activating enzyme CYP27B1 in ejaculated spermatozoa were significantly greater than those in cauda epididymal spermatozoa, while the expression of vitamin D receptor and deactivating enzyme CYP24A1 was significantly higher in high-motility spermatozoa than in low-motility ones (Yao et al., 2018). A study in mice provided evidence that mice fed a vitamin D deficient diet (<25 IU vitamin D_3/kg) in early life had reduced testicular weight, sperm quality and percentage of mature seminiferous tubules, as well as suppressed testicular germ cell proliferation, compared to their counterparts fed a standard chow diet (containing 1000 IU vitamin D_3/kg; Fu et al., 2017). The authors also reported that lower fertility and less live foetuses were found when males and females were both from vitamin D deficient diet groups (Fu et al., 2017).

A summary of effects of vitamin D deficiency on reproductive performance reported in scientific literature is shown in Table 1-2. Mumford et al. (2018) reported that increasing vitamin D concentrations before conception reduced the risk of pregnancy loss. Other studies of pregnant women showed that vitamin D deficiency (serum 25(OH)D<37.5 nmol/l, Gernand et al., 2013; serum 25(OH)D<20 ng/ml, Wang et al., 2018) could result in babies born with lighter birth weight and lower head circumference than those born from vitamin D sufficient counterparts, while placental weight and the ratio of placenta to foetal weight was not affected by maternal vitamin D status (Gernand et al., 2013; Wang et al., 2018). These outcomes were similar to the findings of a meta-analysis of 13 cohort studies (Chen et al., 2017). In contrast, a study of multi-ethnic pregnant women indicated that there was no significant association between maternal vitamin D concentrations and neonate birth weight or head circumference (Eggemoen et al., 2017). A recent study of feral Soay sheep in the St Kilda archipelago at 58°N in the North Atlantic Ocean, where daily sunlight levels are low, showed that ewe 25(OH)D status was positively associated with the number of lambs reared to one year old (Handel et al., 2016).

Table 1-2. Effects of vitamin D deficiency on reproductive performance.

Effect	Species	Reference
Pregnancy loss	Human	Mumford et al., 2018
Lower birth weight and shorter head circumference	Human	Gernand et al., 2013; Chen et al., 2017; Wang et al., 2018
No effect on birth weight and head circumference	Human	Eggemoen et al., 2017
Number of lambs reared	Sheep	Handel et al., 2016

1.7.5 Environmental conditions and ewe reproductive performance

Harsh environmental conditions, as stressors, can have adverse effects on ewe reproductive performance. A study reported in 1927 indicated that number of rainy days is an important climatic factor influencing barren rate and lambing percentage in BF ewes (Nichols, 1927). A negative effect of rain (6 hours per day) from 17 days prior to mating to 25 ± 3 days post mating on ovulation rate and early embryo mortality was also reported for BF ewes (Griffiths et al., 1970). Even when the number of rain days was shortened (from day 1 or day 12 of the post-synchronous cycle to mating), the incidence of delayed onset of oestrus (silent oestrus and failure to ovulate) was higher in the environmentally stressed ewe group, compared to an unstressed ewe group ($P<0.01$; Doney et al., 1973). Under the conditions demonstrated by Griffiths et al. (1970) but from the 13th day to the end of the oestrous cycle, the mean ovulation rate of BF ewes was 1.54 instead of 1.96 per ewe (stressed *vs.* unstressed; Doney et al., 1976). Thus extreme weather conditions around mating can affect the onset of oestrus and ovulation rate and result in increased barren rate and lower lambing percentage.

1.8 Lamb survival/mortality

High lamb mortality rate is one of the major concerns for animal welfare and farm profitability. Pre-weaning lamb mortality levels found in the literature varied from 7% to 31% worldwide (Yapi et al. 1990; Chaarani et al. 1991; Green & Morgan 1993; Binns et al. 2002). Around 50% of these lambs tend to die within 24 hours after birth (Dwyer, 2008b). The main causes of neonatal lamb death are commonly birth trauma during the lambing process; poor ewe and lamb bonding that results in

lambs dying from starvation and hypothermia; infectious diseases; and other causes, such as congenital malformation and predation (Dwyer et al., 2016). Other factors, including light or heavy birth weight, litter size, sex, and dams being young or inexperienced can also lead to high lamb mortality (Chniter et al., 2013; Dwyer et al., 2016).

One single important factor for lamb survival is ingestion of adequate colostrum (Dwyer et al., 2016). Ewe colostrum comprises approximately 10 to 13% fat, 2 to 3% lactose and 7 to 10% non-immunoglobulin protein (Banchero et al., 2015). In an environment where the temperature is between 0°C and 10°C, a lamb requires 280 ml colostrum/kg weight to provide enough energy for its survival, within the first 18 hours of postnatal life. In one study of Santa Ines sheep, the results suggested that, to ensure adequate passive immune transfer, a minimum of 30 g of immunoglobulin G (IgG) should be consumed by the neonatal lamb in the first 24 hours after lambing (Alves et al., 2015). A later study concluded that the quantity of colostrum available during the first 18 hours postpartum can be influenced by lamb birth weight, ewe age, breed and ewe live weight change in late pregnancy, while IgG content can be influenced by breed, lamb birth weight, gestation length and BCS change in late pregnancy (Campion et al., 2019). Twin-bearing ewes secrete more colostrum (Banchero et al., 2015) with higher protein and lactose concentrations (Rosales Nieto et al., 2015) than single-bearing ewes, but the onset of lactation is often slower and the yield of colostrum per lamb is not as much as that from single-bearing ewes (Banchero et al., 2015).

Despite more colostrum being secreted by twin-bearing ewes compared to single-bearing ewes, lambs born in larger litters have lower birth weights with less energy reserves and higher ratios of surface area to birth weight, which render them more likely to die from starvation and hypothermia than singleton lambs (Chniter et al., 2013; Dwyer et al., 2016). Additionally, male lambs stand up more slowly and take a longer time to suck (Dwyer et al., 2005), and they show less strong attachment to their mother than their female counterparts (Gaudin et al., 2015).

The parity of the ewe also has impacts on lamb survival. For hill sheep breeds, such as BF, multiparous ewes prefer to seek isolation before onset of parturition compared with primiparous ewes, which could reduce fearfulness and disturbance

from others during parturition (Alexander et al., 1990). The reduced psychological stress during parturition might subsequently improve maternal behaviour post lambing (Dwyer, 2014). Additionally, colostrum secreted by multiparous ewes contains higher IgG concentration than those secreted by primiparous ewes (Higaki et al., 2013). Moreover, multiparous ewes spend more time than primiparous ewes looking towards and staying together with their own lambs (Romanov breed); for lambs, those born to multiparous ewes, compared to lambs born to primiparous ewes, tend to stay closer to and longer with their own mothers than other mothers (García y González et al. 2015). Lambs born to multiparous ewes also show better neonatal behaviours (stand up and reach udder more quickly) than those born to primiparous ewes (Dwyer et al., 2005). These behavioural and physiological characteristics of multiparas and their lambs are important for enhancing lamb survival.

1.9 Project aims and structure of the thesis

Scottish hill sheep farming sector has an important role for maintaining environmental, economic, and social sustainability in rural areas. However, most of these farming systems are not financially viable without support. Genetic selection to improve hill sheep breeds or breed substitution in hill farming systems might improve flock productivity via improved ewe performance and better lamb survival. These strategies could help to maintain sustainability of hill sheep farming systems in Scotland. The aim of this PhD project was to investigate the effects of a hill environment on the productivity and reproductive performance of a lowland/upland sheep breed (Lleyn) and to compare that breed's performance with the performance of a typical hill sheep breed (BF), the latter comprising two different genetic lines (genetically unimproved and genetically improved; detailed in Section 2.3.2), when all three lines were farmed together on a Scottish hill farm.

In order to investigate the hypothesis that Lleyn ewes would outperform/equal production levels of their BF counterparts from two different genetic lines, in Scottish hill conditions, the following objectives were addressed:

1. In Scottish hill conditions, Lleyn ewes would have higher litter sizes, heavier lamb birth weights and heavier lamb weaning weights than their BF counterparts, with comparable colostrum quality and lower lamb mortality

rate. The nutritional status of Lleyn twin-bearing ewes in late pregnancy would not be compromised in such farming conditions, comparing to BF flockmates.

2. Lleyn ewes would have higher vitamin D concentrations than their BF flockmates (unimproved and improved), that would enhance their reproductive performance.

To address these objectives, Chapter 2 of the thesis presents the comparison of the performance of Lleyn ewes with those of BF from two different genetic lines, farmed together between November 2012 and October 2015, under a moderately harsh hill management system. Several possible reasons that might lead to different performance among the three genetic lines were also investigated in 2015, i.e. pre-lambing metabolic profile, colostrum quality, post mortem examination, and grazing behaviour. Chapter 3 then compares the performance of Lleyn ewes with those of BF, the latter again from the two aforementioned lines, farmed together between November 2015 and October 2017, when different management systems were employed to test the genetic lines under a more extensive hill management system. Further investigations in this study phase included pre-lambing metabolic profiles, colostrum quality, post mortem examination and measurement of ewe external pelvic width.

In order to investigate vitamin D status in sheep, Chapter 4 presents the development of a high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method for the determination of vitamin D status in ewe serum samples, because this vitamin has been reported to influence ewe reproductive performance. Based on these results, Chapter 5 then compares the vitamin D status among the three ewe genetic lines, and examines the effect of ewe pre-mating vitamin D status on ewe reproductive performance, i.e. number of lambs born or weaned per ewe and lamb birth weight. Chapter 6 discusses the findings of the previous chapters and draw overall conclusions.

Chapter 2: Investigation of reproductive performance of Lleyn and Scottish Blackface ewes managed under a conventional or a precision livestock farming system

2.1 Summary

Hill sheep farming plays an important role in the UK sheep industry. The constraints of low productivity of hill sheep breeds has led to a reduction in the number of breeding ewes in this sector. Solutions, such as genetic selection or farming breed substitutes in hill environment should be investigated urgently, in order to maintain viability of hill sheep enterprises. This chapter examined the reproductive performance of approximately 300 unimproved Scottish Blackface (UBF) ewes, 300 genetically improved Scottish Blackface (IBF) ewes and 300 Lleyn ewes for three entire sheep production years, from November 2012 to October 2015. All the results presented in this chapter were based on the data files of ewes mated with rams of their own genotype for two oestrous cycles, except for the barren rates, which also included the data file from the third oestrous cycles, when ewes were mated with the rams from the opposite breed. The results showed that the barren rate of Lleyns at ultrasound pregnancy scanning was significantly lower than those of UBF and IBF counterparts. Lleyn ewes had significantly higher ($P<0.001$) litter sizes at scanning, lambing and weaning than UBF and IBF ewes (based on ewes mated with rams; scanning: 1.30 *vs.* 1.09 and 1.12; lambing: 1.15 *vs.* 0.92 and 0.97; weaning: 1.03 *vs.* 0.79 and 0.86, respectively). The litter size did not differ significantly between UBF and IBF ewes at any of those three stages ($P>0.05$). When based on per ewe lambed, Lleyn ewes also achieved significantly heavier ($P<0.001$) litter birth weight than UBF and IBF ewes (5.65 *vs.* 5.12 and 5.34 kg). Litters born to IBF ewes were heavier than those born to UBF ewes ($P<0.001$). However, weaned litter weight per ewe that lambed did not differ significantly among the three genetic lines ($P>0.05$).

Several possible causes for the observed differences between genetic lines were investigated. Colostrum quality, as measured by specific gravity using an optical Brix refractometer, was not statistically different among the three genetic lines ($P<0.05$). In late pregnancy, Lleyn twin-bearing ewes might have had higher dry matter intake,

as pre-lambing metabolic profiling showed that mean magnesium plasma concentration of Lleyn ewes was higher than that of both UBF and IBF ewes ($P<0.001$ and $P<0.01$, respectively). In the current study flock, lamb mortality rates for UBF, IBF and Lleyn lambs in the 2015 lambing season were 13%, 13% and 10%, respectively. Post mortem examinations of most dead lambs in the 2015 lambing season showed that the most frequent cause of neonatal lamb death was dystocia (32 out of 76 examined lambs); the incidence of dystocia did not vary significantly among the examined UBF, IBF and Lleyn lambs. Summer grazing observations (seven days) suggested that areas where overall grass quality was better attracted more sheep to graze on, compared to those areas with relative poorer grass quality, but genetic line was not a significant determinant for the number of ewes observed in each sector. Overall, the ewes' reproductive performance across three production years, combined with pre-lambing metabolic profile, colostrum quality measurements, post mortem examination and grazing behaviour observations suggested that Lleyn ewes, traditionally a lowland/upland sheep breed, were as competent as their BF flockmates when co-grazed in a hill environment.

2.2 Introduction

In the UK sheep industry, hill farming is an important sector within the unique three-tiered stratified sheep production system, as it provides breeding females to purebred and crossbred farming systems, and also contributes 27% of total UK lamb carcass production (Conington et al., 2006; Pollott et al., 2006; Quality Meat Scotland, 2016). The negative impacts of the reduction of breeding ewes in the Scottish sheep farming sector (The Scottish Government, 2018a,b; see Section 1.3), particularly in the North West of Scotland (SAC Rural Policy Centre, 2008), has led to investigations for solutions to maintain viability and sustainability of hill sheep farming system in Scotland.

Traditionally, sheep (*Ovis aries*) farmed in extensive hill systems are small, hardy breeds, such as BF, which are reputed to have outstanding survivability and superior mothering capability to facilitate lamb survival in extreme weather conditions. This breed is commonly farmed in a wide range of hill and marginal areas in the UK (Dwyer & Lawrence, 1998, 2005). Although the number of BF

breeding ewes has declined over recent times, it is still the most numerous breed in UK sheep farming systems (Pollott, 2014). Additionally, genetic selection is an applicable tool to improve productivity of sheep farming enterprises. After 8 years of selection using a multi-trait selection index (comprising both ewe and lamb traits; Conington et al., 2006), Scotland's Rural College (SRUC) research flocks achieved better lamb weaning weights (increased by 2-2.5 kg), and, as a result, the overall profitability of the flock was improved (Lambe et al., 2014).

Alternatively, introduction of some breeds which are not traditionally farmed in the Scottish hills may offer advantages over the existing genotypes, such as BF or even genetically selected BF sheep. The Lleyn sheep, that used to be confined to the Lleyn Peninsula, North Wales, is a lowland/upland sheep breed, with the characteristics of being prolific (Section 1.2.2; Ceyhan et al., 2015) and good mothering ability (Houlden, 2013; Lleyn Sheep Society, 2018). However, introducing this breed into a harsh hill environment should be carefully investigated in terms of lifelong performance, survivability and resilience, before any firm recommendations can be made.

During a ewe's reproductive cycle, good nutrition is essential to support her genetic potential in terms of reproductive performance, as nutrients (e.g. glucose, amino acids and essential trace elements) obtained from the diet can influence the secretion and so the expression of the sex hormones (Downing & Scaramuzzi, 1991) and these could consequently affect ewe reproduction in the key reproductive stages (Robinson et al., 2002). Undernutrition during the reproductive cycle can result in compromised follicle growth and development (Downing & Scaramuzzi, 1991), reduced ovulation rate at mating (Nottle et al., 1997), increased embryonic mortality in early pregnancy (Russel, 1991), reduction in placental development and subsequent effects on lamb birth weight and lamb vigour (Robinson et al., 1999; Sen et al., 2013). Moreover, nutritional deprivation during pregnancy can also have detrimental effects on the offspring's reproductive performance (Robinson et al., 2002; Hoffman et al., 2018).

During pregnancy, the energy and protein requirements of ewes increase due to foetal growth and development. The increased requirements for nutrients are small during early and mid-pregnancy, when foetal organogenesis and development of

placenta take place (Robinson et al., 1999; Redmer et al., 2004). In late pregnancy, the foetus grows rapidly, which leads to a dramatically increased requirement for energy and protein (Robinson, 1977). At the same time, the size of the gravid uterus expands expeditiously, and the rumen capacity becomes more and more limited (Sargison, 2007). If dietary intake cannot meet the increasing demand related to foetal development, pregnant ewes mobilise their body reserves. In these circumstances, late pregnant ewes, especially twin- and multiple-bearing ewes, might develop metabolic disorders.

Pregnancy toxaemia (syn. twin lamb disease) is a common metabolic disorder of under-fed twin-bearing or multiple-bearing ewes (affecting 1-2% of ewes in well managed flocks and up to 10% in under-nourished flocks), in late pregnancy (Sargison, 2007). This disease is caused when the increasing energy requirement in late pregnancy cannot be met by dietary intake. When ewes are under negative energy balance, body fat reserves are mobilised to release free fatty acids, which are oxidised to acetyl co-enzyme A in the liver to supply energy. The incomplete oxidation of fatty acids, due to lack of an adequate supply of oxaloacetate in the under-nourished animals, results in production of aceto-acetyl co-enzyme A. This product can be hydrogenated to β-hydroxybutyrate (BOHB) and acetoacetate, or decarboxylated to acetone (Herdt, 1988). Accumulation of these toxic ketones is proportional to the extent of body fat mobilisation. In diagnostic laboratories, these ketones are measured as BOHB to indicate nutritional status in late pregnancy (adequate nutrition: BOHB < 0.8 mmol/l of plasma; moderate undernutrition: 0.8-1.6 mmol/l; severe undernutrition: > 1.6 mmol/l; Andrews, 1997; Sargison, 2008). Prevention of this metabolic disorder is important due to the high cost of veterinary treatment, and the high mortality rate in affected animals (Sargison, 2007). Pre-lambing metabolic profiling, which assesses the plasma status of BOHB, albumin, urea nitrogen (urea N), copper and magnesium, of representative cohorts, in late pregnancy is therefore recommended (Russel, 1984, 1991; Dairy Herd Health and Productivity Service, 2014).

Lamb survival is a major contributor to economic efficiency in hill sheep farming systems, and it also is an important indicator of animal welfare (Dwyer, 2008b; Conington et al., 2015). Pre-weaning lamb mortality commonly ranges from 7% to 31% worldwide (Yapi et al., 1990; Chaarani et al., 1991; Kelly, 1992; Green &

Morgan, 1993; Binns et al., 2002; Sawalha et al., 2007). The majority of these mortalities occur within the first week of postnatal life (Wiener et al., 1983). The main causes of neonatal death are hypothermia/starvation, dystocia, infectious disease and predation (Wiener et al., 1983; Green & Morgan, 1993; Dwyer, 2008a). Lamb birth weight is an important factor affecting neonatal lamb mortality (Yapi et al., 1990; Sawalha et al., 2007), and typically birthweight range has a U-shaped relationship with neonatal lamb death incidence (Sawalha et al., 2007; Dwyer, 2008a). Light lambs with less tissue reserves and low vigour, are more susceptible to hypothermia and starvation (Dwyer & Morgan, 2006), while heavy lambs are more prone to death due to trauma during parturition (Scales et al., 1986). Other factors, such as breed, litter size, lamb sex, dam age and management systems also have effects on lamb mortality (Gunn & Robinson, 1963; Huffman et al., 1985; Binns et al., 2002; Dwyer, 2008b, 2017). Hence, lamb mortality rate can vary between farms and years. Post mortem examination of most dead lambs identifies the primary causes of neonatal lamb death in a particular flock, which in turn can help to mitigate these predisposing risk factors in the future (Holst, 2004).

Neonatal lambs have limited energy reserves, and they are dependent on colostrum for maintaining homeothermy and survival. Ewes' colostrum is rich in functional proteins, immunoglobulins (notably IgG), fat, vitamins and minerals (Ahmadi et al., 2016). Adequate consumption of colostrum is important for newborn lambs to obtain essential nutrients and passive immunity for survival. Failure of acquisition of sufficient colostrum after birth has negative impacts on neonatal lamb survival (Ahmad et al., 2000; Dwyer, 2008b) and lamb growth (Massimini et al., 2006). A large number of factors appear to influence the quality and quantity of colostrum, including breed, genotype, nutrition, BCS at lambing, litter size and the age of the dam (Mellor & Murray, 1985; Gilbert et al., 1988; Mellor, 1990; Banchero et al., 2006, 2015). Colostrum secretion commences a few days before parturition, and then it gradually transitions to milk secretion between 12 and 24 hours post lambing (Mellor & Murray, 1985). Thus, the quality of colostrum declines with the time after parturition (Pattinson et al., 2010; Ahmadi et al., 2016). Based on a prediction obtained from linear regression analysis, the IgG content in the colostrum would drop to nearly zero mg/ml at 23 hours after lambing (Al-Sabbagh et al., 1995). The refractometer is an inexpensive and easy to use on-farm tool (Mettler Toledo, 2014),

which allows quick and accurate estimation of colostrum quality for cattle and goats (Bielmann et al., 2010; Quigley et al., 2013; Castro et al., 2018).

Sheep are social animals and some breeds, such as BF (Lawrence & Wood-Gush, 1988) and South Country Cheviot (Hunter & Milner, 1963), form home ranges after being farmed on the same hill or mountain for many years. A number of factors, including breed, age, sex, matrilineage, type of vegetation and environmental conditions can also influence the distribution of sheep on the grazing pasture or hill region (Hunter & Milner, 1963; Arnold et al., 1981; Dwyer & Lawrence, 1999; Sibbald et al., 2008). Thus, farming different breeds together may lead to an inter-dependent or competitive grazing relationship. If competition of grazing resources occurs between breeds, it might have effects on breed diet and performance which could increase potential risk of exposure to toxic plants, such as bog asphodel (*Narthecium ossifragum*). This plant is common in wet heath and moors and wet acid ground, in the North and West of British hill areas (Clapham et al., 1989), and is believed to be the plant which contains photodynamic agents associated with causing plochteach (hepatogenous photosensitisation). The disease often occurs in lambs when accumulated photodynamic agents damage the liver (Sargison, 2008).

This chapter examined the hypothesis that Lleyn ewes would perform better than unimproved Scottish Blackface (UBF) and improved Scottish Blackface (IBF) ewes, with higher litter size, heavier lamb birth weight and heavier lamb weaning weight, in the moderately extensive Scottish hill conditions. More specifically, the objectives of this chapter were to: *1)*, compare ewe reproductive performance (e.g. litter size, litter/lamb birth weight, litter/lamb weaning weight) and lamb growth rate among the three genetic lines; *2)*, examine nutritional status of twin-bearing ewes in late pregnancy via metabolic profiling; *3)*, assess colostrum quality of twin-bearing ewes among the three genetic lines; *4)*, determine primary causes of neonatal lamb death in the flock, and examine any genetic line differences if possible; and *5)*, observe grazing behaviour of single-bearing ewes and their lambs during summer to determine any genetic line differences.

2.3 Materials and Methods

The data used and generated were part of SRUC's RESAS funded research programme – "Improving the economic and environmental sustainability of extensive hill sheep systems".

2.3.1 Study site

The study was conducted at SRUC Hill & Mountain Research Centre (Kirkton and Auchtertyre farms; Figure 2-1) and at the University of Edinburgh (R(D)SVS) labs. The farms are located between Tyndrum and Crianlarich, Scotland (56ºN, 4ºW). The average annual rainfall of the farm was 2613.8 mm, between 1991 and 2015. The average temperatures are the lowest in January (1°C) and the highest in June and August (15°C; Morgan-Davies et al., 2018).

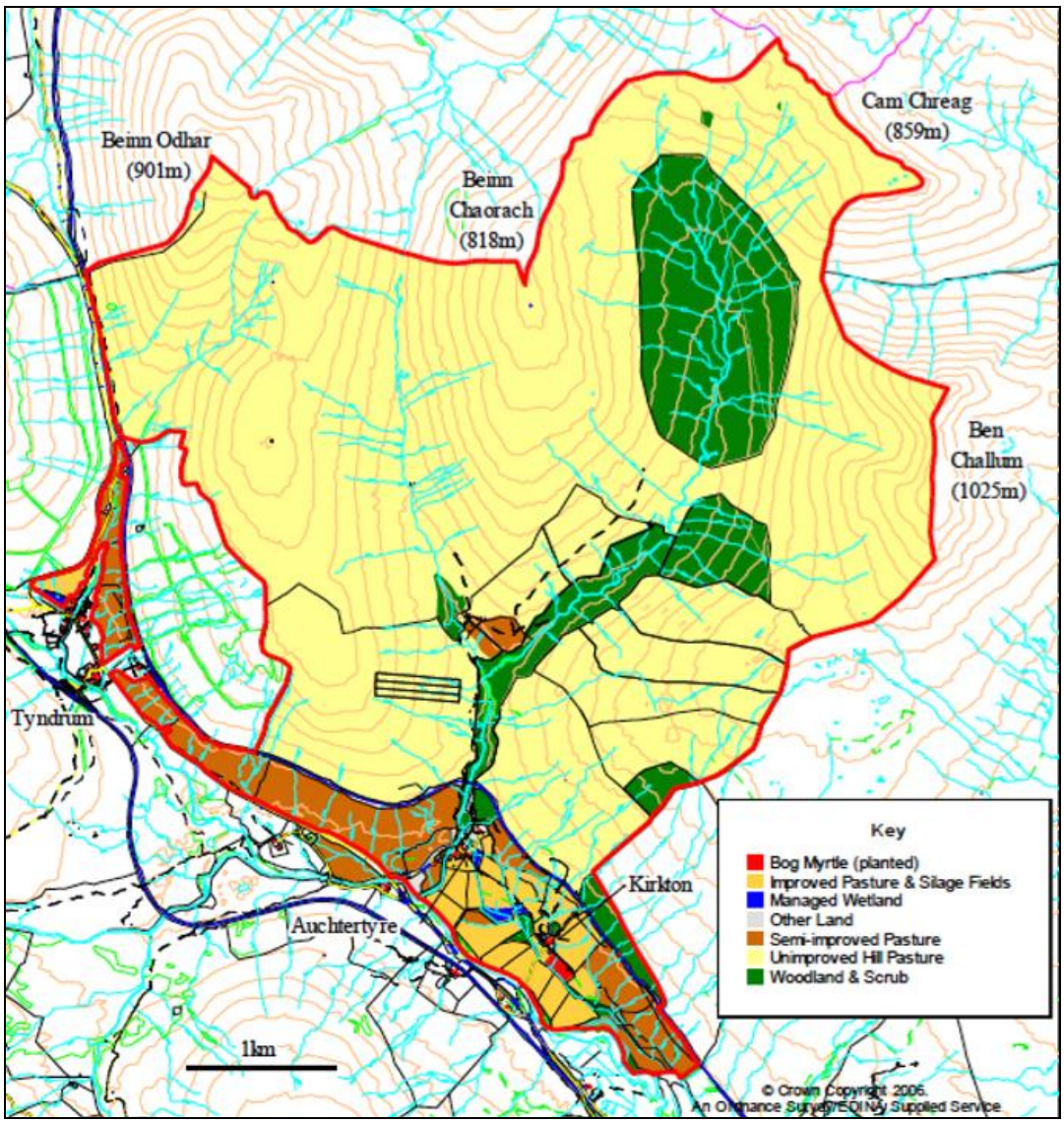


Figure 2-1. Map of Kirkton and Auchtertyre farms.

The SRUC Hill & Mountain Research Centre comprises 2,225 ha of land. These lands are classified into three different types – improved pasture (flat in-bye fields), semi-improved pasture (roughs) and hill ground (unimproved hill pasture; Figure 2-2). The improved pasture consists of 232 ha lands, at an altitude of 170 metres above sea level, where there is potential to make silage and hay. The semi-improved pasture consists of 486 ha of non-fertilised fenced permanent pastures, with a mean altitude of 300 metres, where the grass quality is lower than that in the improved pastures. The hill ground consists of 1,482 ha of unfenced rough grazing areas, with an altitude ranging from 300 to over 1,000 metres, where the grass quality is poor (Morgan-Davies, 2013). The farms are operated in much the same way as Scottish commercial hill farms.

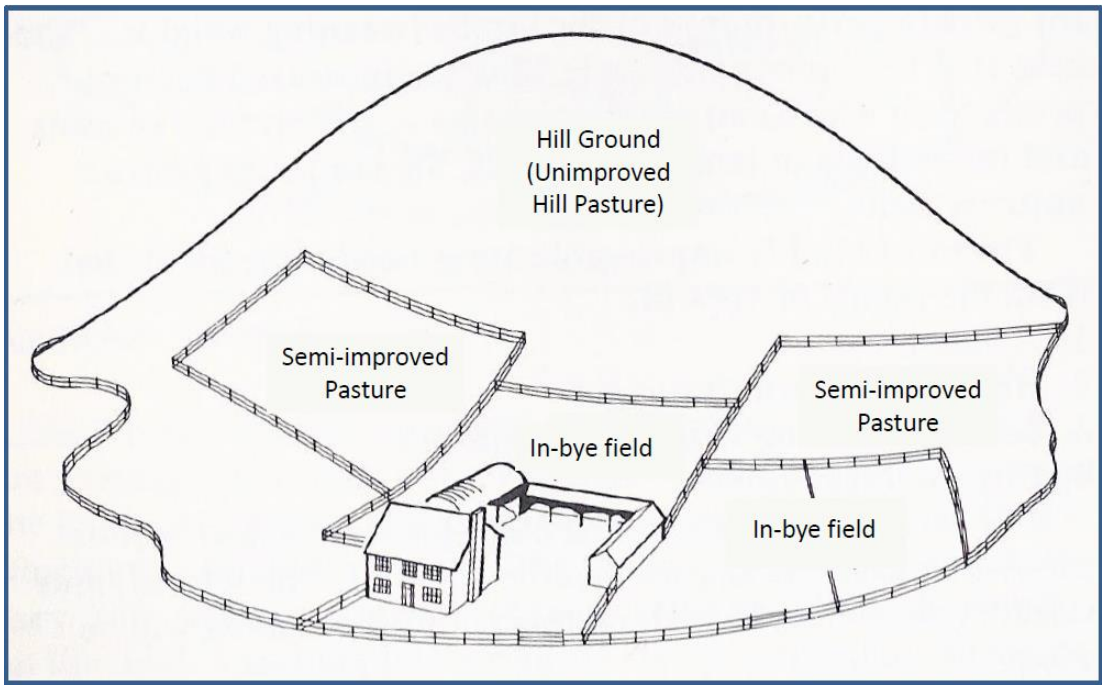


Figure 2-2. Representation of land structure of a hill farm (adapted from Speedy, 1980).

2.3.2 Animals and management systems

Ewes retained in the Kirkton flock were used for investigation of ewe and lamb performance. The Kirkton flock comprised approximately 300 UBF ewes, 300 IBF ewes and 300 Lleyn ewes. The UBF ewes had been selected in each generation to remain close to the average genetic merit in the flock before genetic selection commenced in 1998, while IBF ewes were a lineage progressively selected on a multi-trait index for superior genetic merit in both ewe and lamb traits. The traits selected for in the index were: mature weight, longevity, number of lambs reared, number of lambs lost, maternal weaning weight (total litter weight weaned by the ewe) and fleece weight for ewes; and weaning weight (at average age of 17 weeks), carcass weight, carcass fat class and carcass conformation score for lambs (Conington et al., 2006; see Appendix 2-1 for the genetic divergence between these two lines over 13 years). The Lleyn ewes had been selected for high genetic merit since 2010 using the Carcass+ Index (which aims to identify sheep with superior breeding potential for maternal ability, lamb growth and carcase quality) as part of the Signet Sheepbreeder performance recording service (www.signetfbc.co.uk/sheepbreeder/).

In 2011 pre-mating, ewes were assigned, management-wise, into either a Conventional Livestock Farming system (CON) or a Precision Livestock Farming system (PLF), balanced in terms of genotype, age, breeding group, weight, litter size and sire family. Ewes remained in the same management system for the rest of their lifetime or until November 2015. These two management systems had different criteria for winter feeding, worming and culling (Table 2-1; Morgan-Davies et al., 2014). Single sire mating groups were applied in the flock as a requirement of being part of a performance recorded breeding scheme (Morgan-Davies et al., 2018).

Table 2-1. Management protocols for the CON and the PLF systems (Morgan-Davies et al., 2014).

	CON system	**PLF system**
Winter feeding	Based on BCS and the number of expected lambs (after ultrasound scanning in February)	Based on percentage of weight change and the number of expected lambs (after ultrasound scanning in February)
Worming of lambs	Whole flock approach based on pooled faecal egg counts	Targeted Selective Treatment with anthelminthic based on weight change of individuals
Flock longevity	Breeding sheep culled at a maximum age of 5.5 years	Sheep culled on individual fitness criteria, irrespective of age

From late-November in each production year (Figure 2-3), ewes were joined with rams selected from their own genotype category for two reproductive cycles of 17 days, when rams were then replaced by the rams of the opposite breed for an additional cycle. Ewes were ultrasound pregnancy-scanned in mid-pregnancy (13^{th}/20^{th}, 10^{th}/11^{th} and 10^{th}/11^{th} February for 2013, 2014 and 2015, respectively), for examining pregnancy status and foetal numbers. Two winter supplementary feeding levels, either “standard” or more generous “corrective”, were supplied in two phases to help meet ewes’ intake requirements (Table 2-2; Wishart et al., 2015). The first winter feeding phase was during mid-pregnancy from early January (8^{th}/9^{th}, 7^{th}/8^{th}/9^{th}/10^{th} and 8^{th} January for 2013, 2014 and 2015, respectively) to scanning, while the second winter feeding phase was during late pregnancy from scanning to lambing, which started in early or mid-April (12^{th}, 2^{nd} and 4^{th} April for 2013, 2014 and 2015, respectively). The decision, on which feeding level each ewe would receive in each phase, was based on the individual ewe’s live weight, BCS and pregnancy diagnosis (Wishart et al., 2015).

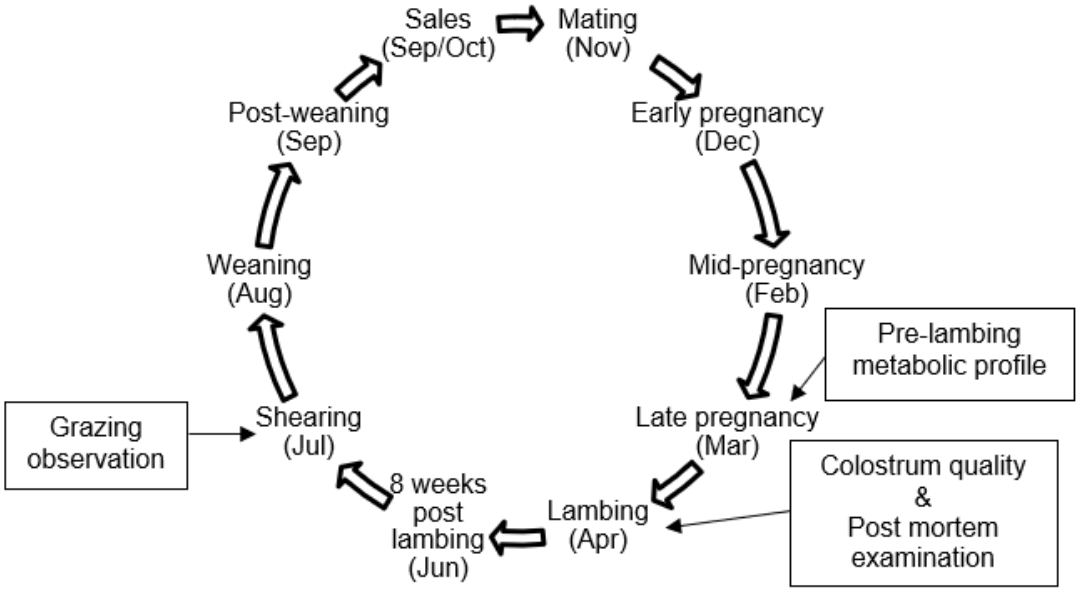


Figure 2-3. An entire sheep production year with main sheep handling events. The boxes indicate the times when additional experiments were conducted in the 2014-2015 production year.

Table 2-2. Criteria for allocating ewes into winter supplementary feeding groups (standard vs. corrective) by management systems (Wishart et al., 2015). EID refers to electronic identification number.

		Standard feeding level	**Corrective feeding level**
CON system	Shepherd determined, based on level of condition and fitness	Ewe in good level of condition and fitness for current stage of pregnancy	Ewes in less than ideal level of condition and fitness for current stage of pregnancy
PLF system	EID weigh-crate and drafting determined, based on weight change	Ewe maintained or put on weight since pre-mating	Ewes lost weight since pre-mating

During lambing, single-bearing ewes managed under both systems were kept in the semi-improved pastures. Twin-bearing ewes were kept on the improved pastures during the daytime and were in a shed overnight, whereas triplet-bearing ewes were kept in the shed. After lambing, ewes that gave birth to singletons were farmed in semi-improved pastures or hill ground with their lambs, whereas ewes that gave birth to twins were farmed in either improved pastures or semi-improved pastures with their lambs, depending on grass availabilities in those areas.

All the experiments were conducted in accordance with the UK legislation under the Animals (Scientific Procedures) Act 1986. The experimental protocols involving animals were approved by the SRUC Animal Welfare and Ethical Review Body.

2.3.3 Ewe and lamb performance

In each sheep production year, ewes were weighed and condition scored using a 5 point scoring system (Russel et al., 1969), to a 0.25 score level of precision, at pre-mating, mid-pregnancy scanning and pre-lambing. Lambs were tagged within 24 hours after birth, and data in terms of weight, sex, litter size, lambing difficulties, ewe maternal behaviour (if lambing outdoors), fostering details and death were recorded. Lambs were then weighed at marking (average 8 weeks old, at end of June) and weaning (average 17 weeks old, at end of August). The records for individual health and group treatments were also kept.

2.3.4 Pre-lambing metabolic profile (March 2015)

Twenty ewes per genotype were selected from healthy, 3-5 year old (at lambing) twin-bearing ewes from the Kirkton flock, based on scanning results. Selected ewes were balanced in terms of management system, feeding level and ewe BCS (Table 2-3). On the 18th March 2015 (late pregnancy), when ewes were gathered for routine clostridial disease vaccination, blood samples were taken via jugular venepuncture into 6 ml green-top blood collection tubes with interior lithium heparin spray-coating (BD, Plymouth, UK). Samples were kept on ice for transportation to the Dairy Herd Health & Productivity Service (commercial lab; Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Easter Bush Campus) for analysis. The plasma concentrations of BOHB, albumin, urea N, copper and magnesium were determined using an IL ILab 650 Chemistry Analyzer (Diamond Diagnostics Inc., USA), within approximately 30 hours after sampling.

Table 2-3. Distribution of twin-bearing ewes selected by management system and second winter feeding level, for pre-lambing metabolic profile in 2015.

	Management system			
	CON		**PLF**	
Feeding level	**Standard**	**Corrective**	**Standard**	**Corrective**
UBF	5	5	5	5
IBF	5	5	5	5
Lleyn	5	5	4	6

Only the data for ewes that gave birth to twins and lambed within 25-46 days after blood sampling were statistically analysed. This narrowed time scale could better match recommended 'ideal blood sampling time' (Dairy Herd Health and Productivity Service, 2014) and enabled more valid comparisons with reference ranges. The statistical analyses included 49 ewes (17 UBF, 16 IBF and 16 Lleyn) in 2015.

2.3.5 Colostrum quality in the 2015 lambing season

During lambing (between the 14th of April and the 4th of May 2015), 93 colostrum samples (24 UBF, 25 IBF and 44 Lleyn) were collected from twin-bearing ewes. Ewes' neckbands, which corresponded to their electronic identification (EID) numbers, were recorded in order to identify their genetic lines, management systems, and winter feeding levels. The intervals between lambing and sampling were estimated, when feasible, by the shepherd. Among these samples, sixty-three were freshly examined, while 30 (10, 8 and 12 samples collected from UBF, IBF and Lleyn twin-bearing ewes, respectively) were frozen after sampling, and were examined later after thawing at room or ambient temperature.

Colostrum quality, expressed as specific gravity (SG) was determined using an optical Brix refractometer. The instrument was calibrated before lambing at the laboratory in the Royal (Dick) School of Veterinary Studies. Each colostrum sample was diluted with tap water to create a 1 in 10 dilution, using a 1 ml or 5 ml syringe. The diluted colostrum sample was dropped onto the refractometer's prism, to provide an immediate reading within the range of the instrument (1.000 to 1.050).

Data for 51 colostrum samples that were collected within 16 hours post lambing (estimated; mean: 4.9 hours; range: 0.33 to 16 hours), were statistically analysed. Among these 51 samples, 48 samples were tested freshly within 2 hours after sampling, while three samples (two UBF and one Lleyn samples) which were after undergoing one freeze-thaw cycle were examined within 48 hours after sampling.

2.3.6 Post mortem examination in the 2015 lambing season

Lambs that died within one week of birth, including stillborn and casualties, but not those showing any signs of abortion, were examined to determine the primary cause of neonatal lamb death in the flock.

Post-mortem examination followed the procedures demonstrated by McFarlane, (1961). Lamb identification, sex, breed, weight and the external appearance of the lamb were recorded. The external observation of a lamb included the status of fleece (wet or dry; presence or absence of meconium stain; Figure 2-4B), status of navel cord (wet or dry; Figure 2-4A), status of eyes (normal or sunken; Figure 2-4C), status of the hooves (Figure 2-5), any subcutaneous oedema of head or neck, and sign(s) of predation (wound inflicted on the body or loss of eye(s); Figure 2-4D). Information obtained from shepherds or technicians, such as congenital abnormality, was also recorded.

After weighing the lamb, the skin under the neck was reflected using a scalpel, and any oedema or subcutaneous injury around the neck or shoulder (Figure 2-6A) was observed and noted. The thyroid glands were removed and weighed (Figure 2-7), the ratio of the weight of thyroid glands to the body weight was calculated. An incision was made through the cartilaginous sternocostal joints from the thoracic cavity to the abdominal cavity. The internal organs of the dead lamb were then examined. The metabolic status of the pericardial and the perirenal brown adipose tissue (Figure 2-8) was assessed. The lung aeration (Figure 2-9) and the state of liver (normal or ruptured; Figure 2-6E) were recorded. The presence or absence of clotted milk in the abomasum, of any clotted or free blood in abdominal cavity (Figure 2-6B or Figure 2-6C, respectively) and of any clotted blood in the umbilical vessel (Figure 2-6D) was determined and recorded.

Figure 2-4. External observation of lambs. Photo A shows a lamb with dried fleece and dried navel cord. Photo B shows a lamb with meconium staining on its fleece and hind legs. Photo C shows a lamb with sunken eyes, which indicates that this lamb was dehydrated. Photo D shows a lamb with a puncture wound on its abdomen.

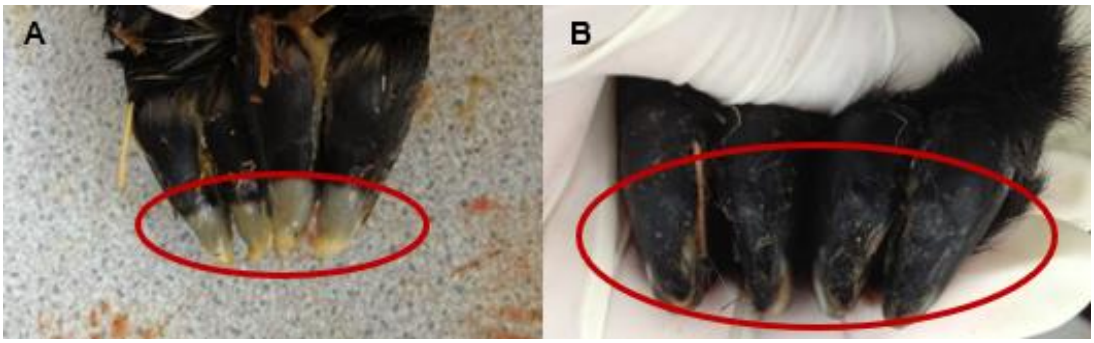


Figure 2-5. Solar views of the feet. Photo A indicates that the lamb had not walked as the membranes were not worn. Photo B indicates that the lamb had walked as the membranes were worn.

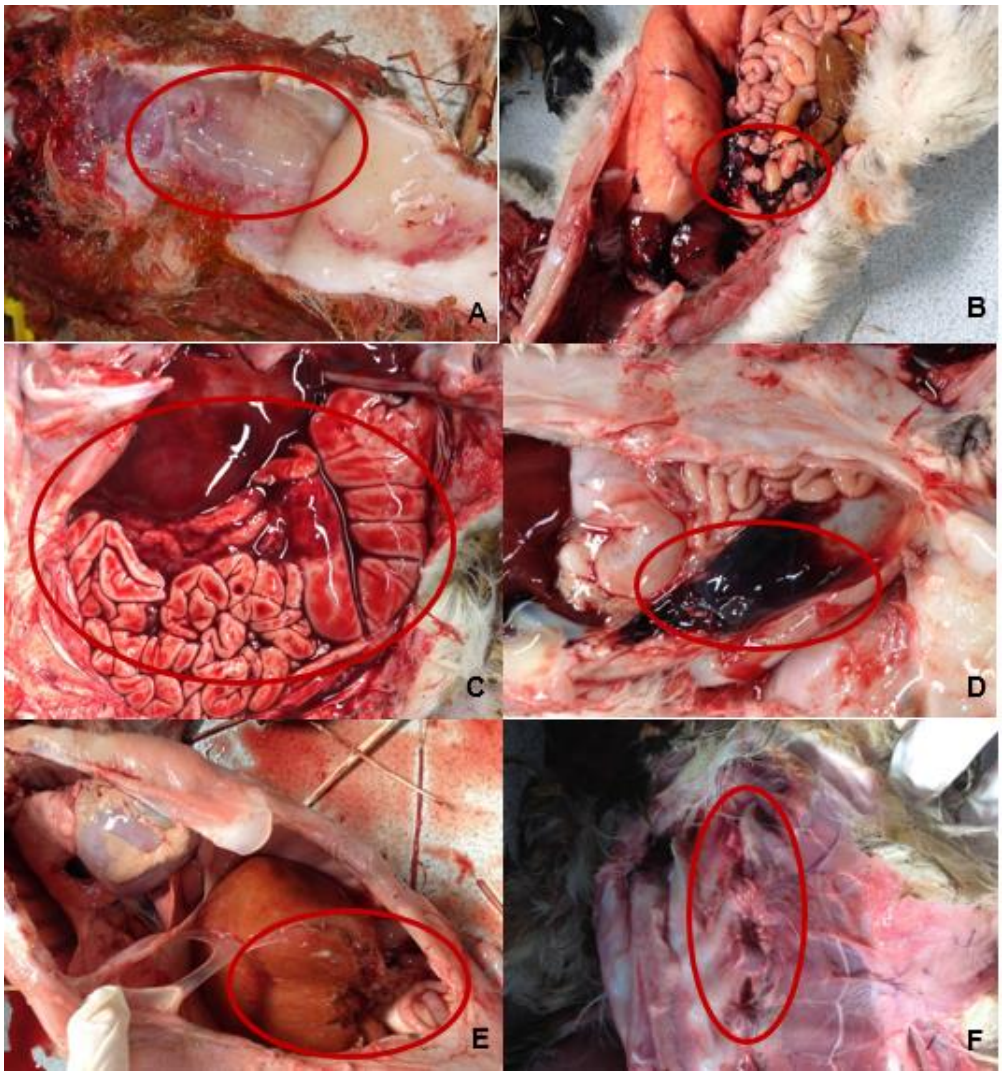


Figure 2-6. Signs of dystocia. Photo A shows oedema under the neck. Photo B shows clotted blood in abdominal cavity. Photo C shows free blood in abdominal cavity. Photo D shows clotted blood in umbilical vessel. Photo E shows liver rupture. Photo F shows rib fractures.

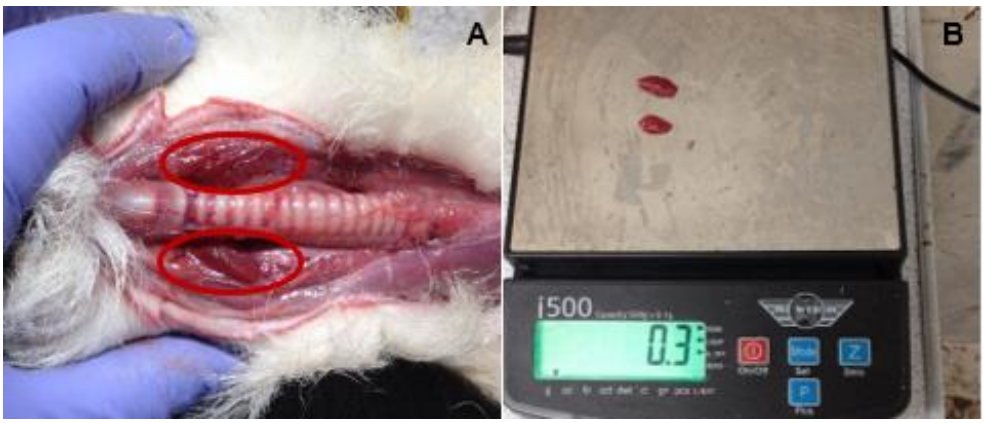


Figure 2-7. Thyroidectomy and weighing of thyroid glands.

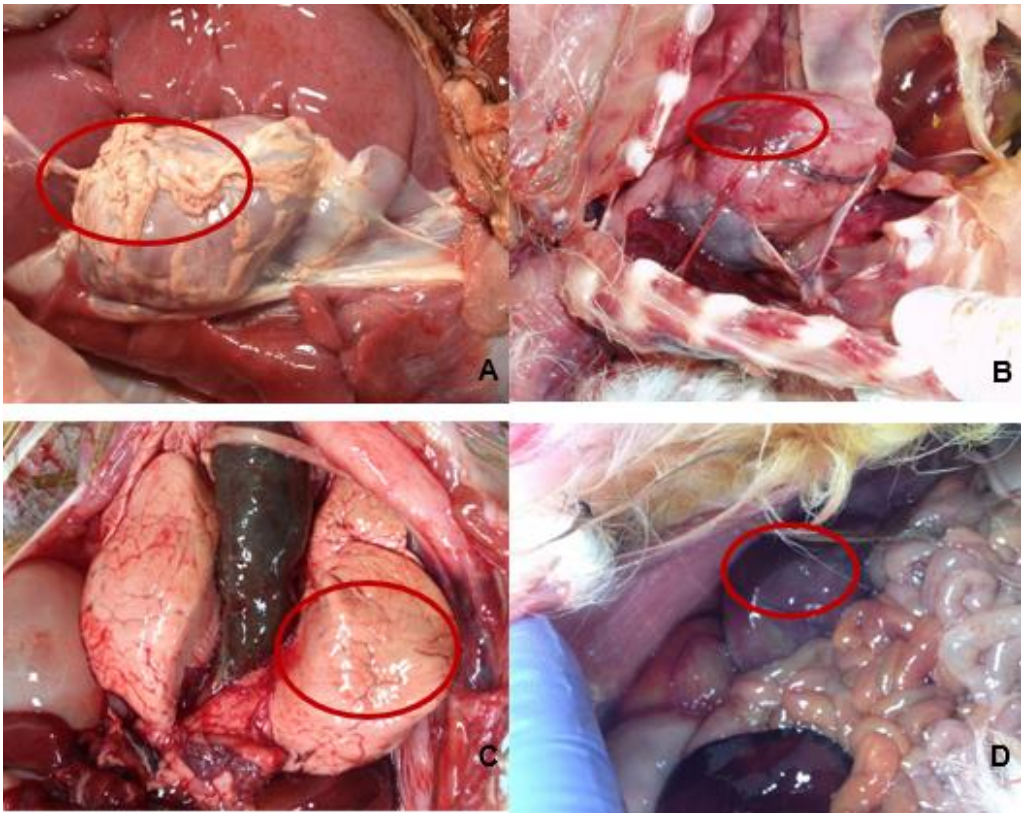


Figure 2-8. Status of brown adipose tissue. Photo A shows non-metabolised pericardial brown adipose tissue. Photo B shows metabolised brown adipose tissue. Photo C shows non-metabolised perirenal brown adipose tissue. Photo D shows metabolised brown adipose tissue.

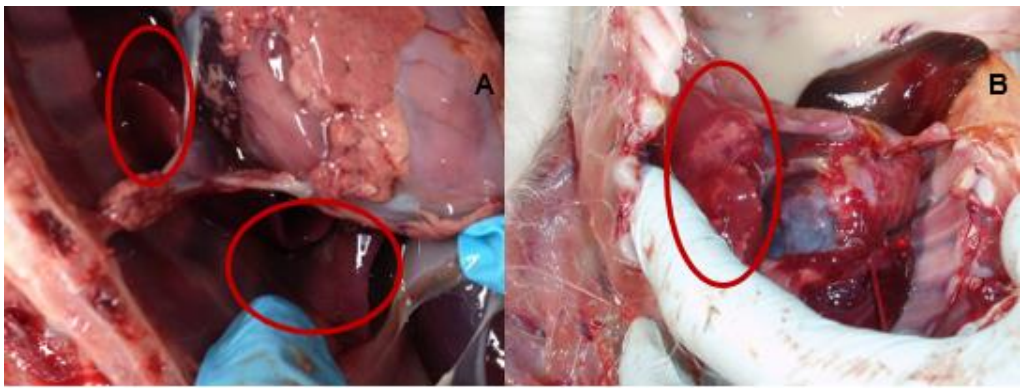


Figure 2-9. Status of lung aeration. Photo A shows non-aerated lungs. Photo B shows aerated lungs.

The key features used in diagnosis:

- *Starvation/Hypothermia*: dead lambs in this category show the following features: low birth weight; aerated lungs; worn soft foot membranes;

absence of clotted milk in the abomasum; pericardial and perirenal brown adipose tissue fully or partially metabolized (according to the weather conditions).

- *Dystocia*: dead lambs in this category show the following features: heavy birth weight, meconium stain on fleece, and/or subcutaneous oedema at neck or head, and/or free or clotted blood in the abdominal cavity, and/or liver rupture, and/or clotted blood in the umbilical vessel.
- *Other causes*: this category includes lambs that died of infection (e.g. watery mouth), predation, abnormality or accident (e.g. ewe had lain on lamb).

2.3.7 Grazing behaviour observation 2015

After the handling event when lambs were 8 weeks old, 55 single-bearing ewes (17 UBF ewes and 20 IBF ewes and 18 Lleyn ewes) and their lambs were introduced into one of the fenced hill areas at Auchtertyre ("The Meall"; see Figure 2-10) on the 23rd of June 2015, for grazing behaviour observation (conducted between the 3rd and 31st July). The ages of the ewes used in the experiment were 2 to 5 years old for UBF ewes, 2 to 6 years old for IBF ewes, and 2 to 8 years old for Lleyn ewes. For identification in the field observation, UBF and IBF ewes were sprayed with bloom dip to dye their wool orange and brown, respectively, while Lleyn ewes were kept with their original fleece colour. This experiment was initially designed for observation of ewe grazing behaviour, so lambs were not sprayed with bloom dip to dye their wool. On the 20th of July, when ewes were brought down for shearing, seven ewes (one UBF, three IBF and three Lleyn) and their lambs were removed from the observation group, as the lambs had plochteach.

Nine days of field observations, two days per week, were performed from a vantage point on the slope opposite The Meall. The actual observation days were decided according to weather forecast for good visibility. The locations of ewes and lambs were recorded hourly from 9 am to 4 pm, by two student observers. The first observation day was conducted as a trial, and another observation day (21st July 2015) was performed on the day after a sheep handling event, so more sheep were observed gathering on the left of that hill side. These two days of grazing observation data were excluded from data used for statistical analysis.

The hill area used for grazing observations was mapped to indicate contrasting sectors A to N on plexiglass (Figure 2-10). These sectors were based on the colour of the vegetation patch on a colour photograph, taken on the 16th of June 2015 (Appendix 2-2). Of these sectors, Sectors B and J were fenced off and ewes could not access them. At each observation: *1),* the hill area being grazed was photographed; *2),* weather conditions were recorded; *3),* an acetate sheet was placed over the plexiglass map, and the locations of visible ewes and lambs were marked on the acetate sheet with different coloured permanent marker pens indicating age (ewe or lamb) and genotype. Five different colours were used to indicate ewes of different genotypes and lambs of different breeds (Table 2-4).

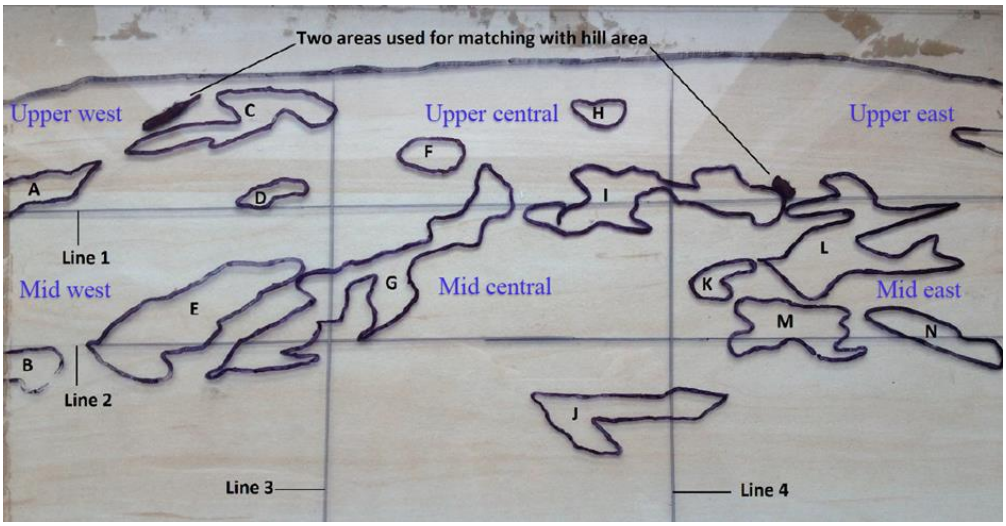


Figure 2-10. The plexiglass map used in sheep grazing observation, showing sectors A to N.

Table 2-4. Pen colours used for marking sheep locations on acetate sheet.

	Genotype	Pen colour
Ewes	UBF	Orange
	IBF	Blue
	Lleyn	Dark green
Lambs	BF	Pink*
	Lleyn	Light green

* lambs not specific to either BF genotype.

The plexiglass map was further divided into nine zones using Lines 1 to 4 (Figure 2-10), for increased accuracy when transferring observation data into an excel database. The locations of ewes and lambs were recorded according to the 'sector (if within the sector)' or 'zone (if outside of any sector)' where they were observed.

The densities of bog asphodel in sectors C, D, F, H, I and L where more sheep were observed were recorded on the 4th and 14th of September 2015. The density of bog asphodel was estimated by throwing a quadrat (1 x 1 m), randomly three times, in low or middle or high (attitude-wise) areas within each distinctive sector.

2.3.8 Data analysis

Data were collated, and ewes that had lamb(s) fostered on or off and those that had crossbred lambs were removed from the data file. The one exception was the data file used to analyse barren rate at the pregnancy scanning, for which the data file considered all the ewes that were presented at the pregnancy scanning handling event, including those that subsequently gave birth to crossbred lambs or had lambs fostered on or off. The number of records in the data files for ewe and lamb performance are summarised in Table 2-5.

Table 2-5. Summary of sample size for the ewe and lamb performance traits.

Performance trait	**UBF**	**IBF**	**Lleyn**
Number of lambs scanned per ewe mated	690	731	833
Number of lambs born or weaned per ewe mated	700	736	835
Number of lambs weaned per ewe lambed	528	569	710
Barren rate (at scanning)*	790	830	907
Litter birth weight (excluding ewes that did not lamb)	528	569	710
Average lamb birth weight (excluding ewes that did not lamb)	528	569	710
Weaned litter weight (excluding ewes that did not lamb)	528	569	710
Average lamb weaning weight (ewes that weaned lambs)	445	495	644
Lamb growth rate between birth and marking	612	728	909
Lamb growth rate between marking and weaning	585	712	898

*The sample size of barren rate at scanning included all the ewes that were presented at pregnancy scanning.

All the statistical analyses were conducted using GenStat 16 statistical package (VSN International Ltd, UK). Most analyses of ewe and lamb performance among the three genetic lines were investigated using Linear Mixed Model (LMM) via: *1)*, comparing differences in litter size at scanning (based on scanning results, barren ewes included as zero), lambing (ewes that did not lamb included as zero) and weaning (ewes that did not wean lamb included as zero); *2)*, examining differences in litter weight at birth and weaning (excluding ewes that did not lamb) and average lamb weight at birth (excluding ewes that did not lamb) and at weaning (excluding ewes that did not wean lambs); *3),* assessing lamb growth rate (calculated for

average daily gain; g/day) from birth to marking and from marking to weaning. The barren rate at pregnancy scanning (based on scanning results: barren ewe = 0 or non-barren ewe = 1) was analysed using a Generalized Linear Mixed Model (GLMM) with a binomial distribution and a logit link function. The random effects applied in these statistical analysis models were either 'year (in this chapter, it was with three levels: 2013 or 2014 or 2015)' or 'sire EID' and 'year'. The fixed and random effects applied for each response variate are summarised in Table 2-6.

The fixed effects and relevant covariates applied for investigation of ewe performance were genetic line (UBF, IBF or Lleyn), ewe pre-mating weight (to nearest 0.1 kg), ewe pre-mating BCS (to 0.25 score), ewe age (six levels: from 2 to 7+ years old; 7+ years old included 7 and 8 years old ewes), first winter feeding level (five levels: standard for gimmer, standard for mature ewe, corrective for gimmer, corrective for mature ewe or corrective for poor sheep), second winter feeding level (assessed as a covariate; for each individual ewe this was the average weight of feed per head offered to the ewe across the number of days in that second winter feeding phase, which ranged from 0 g to 450 g (34 different feeding levels across the years, litter sizes and ewe weight/condition categories), management system (two levels: CON or PLF), birth litter sex (nine levels, which represented the sex of all the lambs within the litter at birth, for example, for a ewe that gave birth to triplets comprising two male lambs and one female lamb, the birth litter sex would be MaleMaleFemale), lamb age at weaning (days) and weaned litter sex (eight levels, which represented the sex of all the lambs within the litter at weaning, for example, for a ewe that weaned twins, these being one male lamb and one female lamb, her weaned litter sex would be MaleFemale; in this chapter, for ewes that had three lambs weaned, the weaned litter sex levels were MaleMaleMale, MaleMaleFemale, MaleFemaleFemale). The fixed and random effects applied for each response variate are summarised in Table 2-6.

The fixed effects and relevant covariates applied for investigation of lamb performance were genetic line (as above), lamb birth weight (to nearest 0.1 kg), lamb weight at marking (to 0.1 kg), lamb sex (two levels: male or female), litter size at birth (three levels: singleton, twin or triplet), lamb age at marking (days), number of days between marking and weaning, litter size at weaning (three levels: singleton, twin or triplet), dam age (six levels: from 2 to 7+ years old; 7+ years old included 7

and 8 years old ewes) and management system (as above). The fixed and random effects applied for each response variate are summarised in Table 2-6. For lamb growth rate between marking and weaning, lamb birth weight was fitted in the fixed model for LMM analysis, but the model would not converge, so lamb birth weight was dropped from the final model.

The statistical analyses of the plasma concentrations of BOHB, albumin, urea N, copper and magnesium of twin-bearing ewes in late-pregnancy were carried out using Generalized Linear Models (GLM). Genetic line (as above), ewe age (three levels: from 3 to 5 years old), ewe weight (as above) and BCS (as above) at scanning, first winter feeding level (two levels: standard or corrective), second winter feeding level (two levels: standard or corrective), number of days between sampling and lambing, litter birth weight (to nearest 0.1 kg) and management system (as above) were applied in the Maximal Models for all the metabolites investigated. The variables used in the final model for each metabolite were based on the suggestions from stepwise regression and are summarised in Table 2-6. As the second winter feeding level was a significant determinant for the concentrations of BOHB and urea N, the interaction effect of genetic line and the second winter feeding level was tested for these two metabolites using GLM (Table 2-6), in order to determine the differences between the two second winter feeding levels for the three genetic lines.

The data (SG values) for colostrum samples were analysed using GLM, with genetic line (as above), ewe age (six levels: 2 to 7 years old), time between lambing and sampling (to 1 minute or estimated by the shepherd), ewe weight (as above) and BCS (as above) at scanning, first winter feeding level (four levels: standard for gimmer, standard for mature ewe, corrective for gimmer or corrective for mature ewe), second winter feeding level (two levels: standard or corrective), litter birth weight (as above) and management system (as above) being the variables included in the Maximal Model. The variables suggested by stepwise regression for the final model are summarised in Table 2-6.

The effect of genetic line (pure-bred lambs only) on the incidence of lambs dying of dystocia (lamb died of other reasons excepting dystocia = 0 or lamb died of dystocia = 1) was determined using GLMM, fitting a binomial distribution and a logit link function. GLM analysis was used to test the effects of genetic line (as above), dam

age (six levels: from 2 to 7+ years old; 7+ years old included 7 and 8 years old ewes), lamb birth weight (as above), litter size at birth (as above), lamb sex (as above), lambing date (converted to the number of days after official start of lambing date in 2015), ewe pre-mating weight (as above), ewe pre-mating BCS (as above) and management system (as above) on this response variate. Management system was found not to be a significant determinant for lamb death from dystocia, thus it was used as a random effect in the GLMM analysis. The fixed model and random model used in the GLMM for this response variate were based on the suggestion of the stepwise regression (Table 2-6). The birth weights of examined lambs were statistically analysed using GLM. The factors considered in the maximal model were genetic line (as above), dam age (as above), litter size at birth (as above), lamb sex (as above) and cause of lamb death (four levels: dystocia, starvation/hypothermia, other causes, or inconclusive diagnosis). The final model was based on the suggestion of stepwise regression (Table 2-6). Lamb birth weight of the examined lamb was found to be a significant determinant for cause of lamb death, therefore, the interaction effect of genetic line and cause of lamb death was tested using GLM analysis (Table 2-6), in order to determine the differences between different levels of cause of lamb death for different lamb groups.

Statistical analysis of ewe grazing observation data (seven days) was carried out using GLMM, fitting a poisson distribution and a logarithm link function. The GLMM models for number of ewes observed in sectors and in zones are summarised in Table 2-6. In the model, 'Genotypedayhour' combined the genetic line of the ewe observed on a specific observation date and time, for example, UBF 1410; 'sectorcombine' (five levels: sector C, F, H, I or ADEGKLM; the latter was a combination of sectors A, D, E, G, K, L and M, due to low number of ewes observed in those sectors); 'zonecombine' included zone upper_east, upper_central, upper_west and middle (four levels; the latter was a combination of zones mid_east, mid_central and mid_west, due to low number of ewes observed in those zones); 'timeinday' divided observation times into two levels, which were 9:00 to 10:00 and 11:00 to 15:00.

Statistical significance was defined as $P < 0.05$. When model terms were significant, pairwise Student's t-tests were performed to test for significant differences between different levels of each factor.

Table 2-6. Summary of statistical analyses applied, with corresponding type of the analysis and final models with fixed effects and random effects applied for each response variate.

Response variate category	Response variate	Type of analysis	Fixed model for LMM or GLMM / Model to be fitted for GLM	Random model
Ewe performance	Number of lambs scanned per ewe mated	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + management system	Year
	Number of lambs born per ewe mated	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + management system	Year
	Number of lambs weaned per ewe mated	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + management system	Year
	Number of lambs weaned per ewe lambed	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + management system	Year
	Barren rate (at scanning)	GLMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + management system	Year
	Litter birth weight (excluding ewes that did not lamb)	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + (second winter feeding level x year) + management system	Year
	Average lamb birth weight (excluding ewes that did not lamb)	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + (second winter feeding level x year) + birth litter sex + management system	Year
	Weaned litter weight (excluding ewes that did not lamb)	LMM	Genetic line + ewe pre-mating weight + ewe age + first winter feeding level + (second winter feeding level x year) + lamb age at weaning + management system	Year
	Average lamb weaning weight (ewes that had lambs weaned)	LMM	Genetic line + ewe pre-mating weight + ewe age + first winter feeding level + (second winter feeding level x year) + lamb age at weaning + weaned litter sex + management system	Year
Lamb performance	Lamb growth rate between birth and marking	LMM	Genetic line + lamb birth weight + lamb sex + litter size at birth + lamb age at marking + dam age + management system	Sire EID + year

Response variate category	Response variate	Type of analysis	Fixed model for LMM or GLMM / Model to be fitted for GLM	Random model
	Lamb growth rate between marking and weaning	LMM	Genetic line + lamb sex + litter size at weaning + number of days between marking and weaning + lamb age at marking + dam age + management system	Sire EID + year
Pre-lambing metabolic profile	BOHB concentration	GLM	Genetic line + ewe age + second winter feeding level + number of days between sampling and lambing + management system	-
	BOHB concentration[a]	GLM	Genetic line.second winter feeding level + ewe age + number of days between sampling and lambing + management system	-
	Albumin concentation	GLM	Genetic line + ewe BCS at scanning + litter birth weight + number of days between sampling and lambing	-
	Urea N concentration	GLM	Genetic line + ewe age + second winter feeding level + ewe BCS at scanning + management system	-
	Urea N concentration[b]	GLM	Genetic line.second winter feeding level + ewe age + ewe BCS at scanning + management system	-
	Copper concentration	GLM	Genetic line + first winter feeding level + ewe BCS at scanning + litter birth weight + number of days between sampling and lambing + management system	-
	Magnesium concentration	GLM	Genetic line + ewe weight at scanning + litter birth weight + number of days between sampling and lambing	-
Colostrum quality	SG value	GLM	Genetic line + ewe age + time between lambing and sampling + litter birth weight	-
Post mortem examination	Death from dystocia	GLMM	Genetic line + lamb birth weight	Management system
	Lamb birth weight	GLM	Genetic line + litter size at birth + cause of lamb death	-
	Lamb birth weight[c]	GLM	Genetic line.cause of lamb death + litter size at birth	-
Grazing observation	Number of ewes in sectors	GLMM	Genotypedayhour + sectorcombine + (sectorcombine.genetic line) + (sectorcombine.timeinday)	Sectorcombine.day + (sectorcombine.hour)
	Number of ewes in zones	GLMM	Genotypedayhour + zonecombine + (zonecombine.genetic line) + (zonecombine.timeinday)	Zonecombine.day + (zonecombine.hour)

[a]This model was used to test the differences between the two different second winter feeding levels for the three genetic lines.
[b]This model was used to test the differences between the two different second winter feeding levels for the three genetic lines.
[c]This model was used to test the effect on lamb birth weight of differences between different causes of lamb death for the three genetic lines.

2.4 Results

2.4.1 Ewe reproductive performance (Nov 2012 to Oct 2015)

The percentages of ewes in the different litter size categories at birth and weaning are shown in Table 2-7. The unadjusted results in terms of ewe and lamb performance for UBF, IBF and Lleyn are shown in Table 2-8.

Table 2-7. Distribution of ewes by litter size at birth and weaning for UBF, IBF and Lleyn.

		Litter size							
		Birth				Weaning			
Year	**Genotype**	**0***	**1**	**2**	**3**	**0**	**1**	**2**	**3**
2013	**UBF**	24%	50%	26%	0	36%	45%	19%	0%
	IBF	20%	41%	38%	1%	29%	42%	29%	0%
	Lleyn	11%	42%	44%	3%	23%	48%	29%	0%
2014	**UBF**	29%	48%	23%	0%	42%	39%	18%	0%
	IBF	30%	38%	31%	0%	43%	28%	29%	0%
	Lleyn	19%	49%	29%	3%	24%	48%	27%	1%
2015	**UBF**	20%	43%	35%	2%	30%	43%	26%	0%
	IBF	18%	38%	43%	1%	26%	43%	31%	0%
	Lleyn	15%	44%	37%	3%	20%	47%	30%	2%

* percentages in these columns were based on just two oestrous cycles. Some of those ewes became pregnant in the subsequent oestrous cycle. Lower barrenness data in Section 2.4.1.1 reflect this.

Table 2-8. Ewe and lamb performance data (mean ± standard deviation (SD)) recorded for the three genetic lines across three consecutive production years.

Animal performance	Production year	UBF	IBF	Lleyn
Ewe mature weight (kg)*	2012-2015	48.2	49.8	49.1
Replacement rate (%)	2012-2013	26	25	23
	2013-2014	25	25	21
	2014-2015	28	30	32
Ewe pre-mating weight (kg ± SD)	2012-2013	52.0 ± 5.9	54.2 ± 5.8	53.0 ± 5.6
	2013-2014	46.0 ± 6.0	47.7 ± 5.8	47.2 ± 6.2
	2014-2015	52.4 ± 5.6	54.1 ± 6.1	50.0 ± 6.1
Ewe pre-mating BCS (± SD)	2012-2013	2.8 ± 0.3	2.8 ± 0.3	2.8 ± 0.2
	2013-2014	2.9 ± 0.3	2.9 ± 0.3	2.8 ± 0.3
	2014-2015	2.6 ± 0.3	2.6 ± 0.3	2.4 ± 0.3
Ewe scanning weight (kg ± SD)	2012-2013	46.9 ± 5.7	49.4 ± 5.7	48.4 ± 5.5
	2013-2014	47.1 ± 6.2	49.1 ± 6.2	47.7 ± 5.8
	2014-2015	50.6 ± 5.6	52.5 ± 6.1	50.2 ± 6.1
Ewe scanning BCS (± SD)	2012-2013	2.6 ± 0.3	2.6 ± 0.3	2.6 ± 0.2
	2013-2014	2.8 ± 0.3	2.8 ± 0.3	2.8 ± 0.3
	2014-2015	2.9 ± 0.3	3.0 ± 0.3	2.8 ± 0.3
Ewe pre-lambing weight (kg ± SD)	2012-2013	50.0 ± 7.2	53.4 ± 6.8	52.4 ± 6.2
	2013-2014	50.0 ± 7.4	52.2 ± 7.3	52.1 ± 7.0
	2014-2015	52.4 ± 7.9	56.1 ± 8.0	53.5 ± 8.3
Barren rate at pregnancy scanning (%)	2012-2013	8	8	4
	2013-2014	21	23	13
	2014-2015	9	10	7
Ewe mortality (%)	2012-2013	3.7	5.3	7.2
	2013-2014	5.1	3.1	2.9
	2014-2015	2.2	2.1	3.4
Litter birth weight per ewe mated (kg ± SD)	2012-2013	3.4 ± 2.3	3.9 ± 2.4	4.7 ± 2.3
	2013-2014	3.2 ± 2.5	3.6 ± 2.8	4.6 ± 2.8
	2014-2015	4.3 ± 2.7	4.6 ± 2.7	5.1 ± 2.8
Litter birth weight per ewe lambed (kg ± SD)	2012-2013	4.6 ± 1.4	5.0 ± 1.5	5.3 ± 1.6
	2013-2014	4.5 ± 1.6	5.2 ± 1.7	5.7 ± 1.9
	2014-2015	5.4 ± 1.8	5.6 ± 1.8	6.1 ± 1.9
Average lamb birth weight per ewe lambed (kg ± SD)	2012-2013	3.5 ± 0.7	3.4 ± 0.7	3.6 ± 0.9
	2013-2014	3.5 ± 0.8	3.6 ± 0.7	4.1 ±0.9
	2014-2015	3.7 ± 0.7	3.7 ± 0.7	4.2 ± 0.9
Weaned litter weight per ewe mated (kg ± SD)	2012-2013	21.9 ± 19.6	27.3 ± 21.3	28.6 ± 20.8
	2013-2014	21.0 ± 20.9	24.6 ± 24.5	29.9 ± 21.7
	2014-2015	27.7 ± 22.1	29.6 ± 22.1	32.4 ± 23.4
Weaned litter weight per ewe that weaned lamb(s) (kg ± SD)	2012-2013	34.1 ± 13.4	38.2 ± 14.7	37.3 ± 15.4
	2013-2014	36.5 ± 13.8	43.1 ± 15.9	39.5 ± 15.5
	2014-2015	39.8 ± 14.8	40.2 ± 15.3	40.7 ± 18.7

* Ewe mature weight was estimated by standardizing pre-mating weights to a BCS of 2.75 and ewe age to 3 years old (Carson et al., 2001a).

2.4.1.1 Litter size

At ultrasound pregnancy scanning, the barren rate of Lleyn ewes was lower than those of UBF and IBF ewes (P<0.001 and P<0.001, respectively; predicted means:

UBF = 16%, IBF = 20%, Lleyn = 9%), when data were adjusted for ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P<0.001), ewe age (P=0.642), first winter feeding level (P<0.001) and management system (P=0.529). Ewe weight and BCS prior to mating were negatively associated with ewe barrenness. Mature ewes (>2 years old) that received standard feeding level in the first winter feeding period had lower barren rate than those that received corrective feeding level in the same period (P<0.05; predicted means: standard = 9%, corrective = 13%), whereas no such difference was found between gimmers (2 years old) that received standard feeding level and corrective feeding level in the first winter feeding period (predicted means: standard = 17%, corrective = 16%). Ewes fed with standard feeding level and corrective feeding level in the first winter feeding phase had lower barren rate than those (from 2 to 7+ years old) that received corrective feeding level for poor sheep (P<0.001 and P<0.01, respectively; predicted mean: corrective for poor sheep = 22%).

Lleyn ewes had significantly greater litter size at ultrasound pregnancy scanning than UBF and IBF ewes (P<0.001 and P<0.001, respectively), after adjusting for ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P<0.001), first winter feeding level (P<0.001), ewe age (P=0.163) and management system (CON *vs.* PLF; P=0.376; Figure 2-11). The number of foetuses carried per ewe at scanning did not differ significantly between UBF and IBF ewes (P>0.05). Ewe pre-mating weight and pre-mating BCS were positively associated with the number of foetuses carried per ewe at pregnancy-scanning. Mature ewes that received the standard feeding level in the first winter feeding period had significantly greater litter size at ultrasound scanning than those that received the corrective feeding level during the same period (P<0.001), while there was no significant difference in litter size at scanning between gimmers that received the standard and those that received the corrective feeding level in the first winter feeding period.

At lambing, Lleyn ewes had significantly greater litter size than UBF and IBF ewes (P<0.001 and P<0.001, respectively), when ewe age (P=0.072), ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P<0.001), first winter feeding level (P<0.001) and management systems (P=0.433; Figure 2-11) were fitted as fixed effects in the LMM model. There was no significant difference in the number of lambs born per ewe mated between UBF and IBF ewes (P>0.05). Ewe pre-mating

weight and pre-mating BCS were positively associated with number of lambs born in the following spring. Mature ewes fed with the standard feeding level in the first winter feeding phase had greater litter size than their counterparts that were fed with the corrective feeding level (P<0.001), whereas feeding level was not a significant determinant of the number of lambs born among the gimmers (P>0.05).

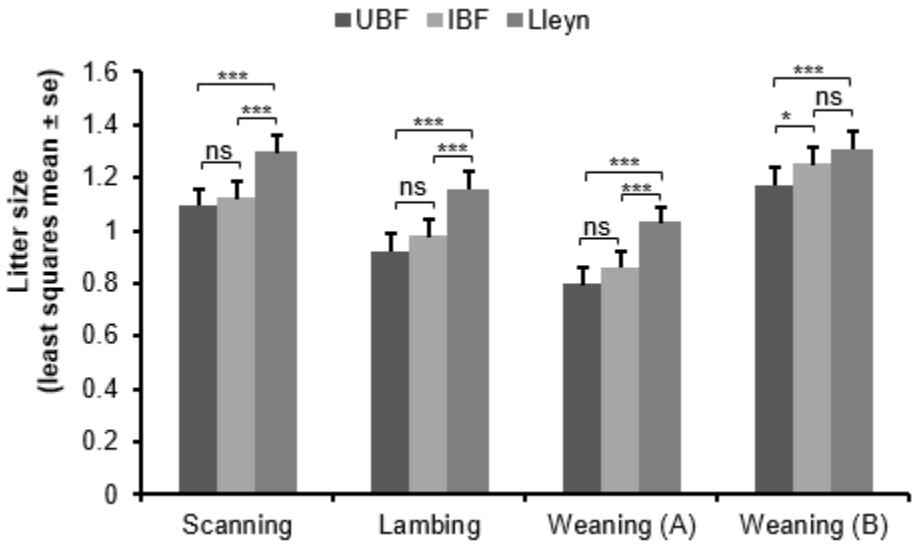


*Figure 2-11. The litter sizes at scanning, lambing and weaning (least squares means ± standard error (se)) for the three genetic lines. Data for Scanning, Lambing and Weaning (A) represent litter size per ewe mated, while data for Weaning (B) represent litter size per ewe lambed. ns indicates not significant (P>0.05); * indicates significance at P<0.05; ** indicates significance at P<0.01; *** indicates the significance at P<0.001. Same symbols for denoting the level of significance are used in all Figures in this chapter.*

After adjusting for ewe age (P=0.002), ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P=0.002), first winter feeding level (P<0.001) and management system (P=0.537), Lleyn ewes had more lambs weaned than UBF and IBF ewes (P<0.001 and P<0.001, respectively; Figure 2-11), per ewe mated. Number of lambs weaned per ewe mated was not significantly different between UBF and IBF ewes (P>0.05). Ewe pre-mating weight and pre-mating BCS were positively associated with the number of lambs weaned per ewe mated in the following summer. Mature ewes that received the standard feeding level in the first winter feeding period had more lambs weaned than their counterparts that received the corrective feeding level, while the first winter feeding level did not have such effect in the group of gimmers (P>0.05). Ewes that were 4 and 5 years old at lambing had more lambs weaned than ewes that were 3 years old at lambing (P<0.001 and P<0.01, respectively). There was no

significant difference in the number of lambs weaned per ewe mated among the other age groups (P>0.05). After removing the ewes that did not lamb from the data set, IBF and Lleyn ewes had significantly more lambs weaned per ewe lambed than UBF ewes (P<0.05 and P<0.001, respectively; Figure 2-11). The number of lambs weaned per ewe lambed did not differ significantly between IBF and Lleyn ewes. Ewe pre-mating weight was positively associated with the number of lambs weaned per ewe lambed (P<0.001). Ewes that were 4 and 5 years old at lambing had more lambs weaned than ewes that were 3 years old at lambing (P<0.01 and P<0.05, respectively). Ewe pre-mating BCS (P=0.178), first winter feeding level (P=0.071) and management system (P=0.866) did not have significant effects on the number of lambs weaned per ewe lambed.

2.4.1.2 Birth weight

Excluding barren ewes, Lleyn ewes had the heaviest litter birth weights among the three genetic lines, followed by IBF ewes and UBF ewes (Figure 2-12), using the LMM model which adjusted for ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P=0.594), ewe age (P<0.001), first winter feeding level (P<0.001), interaction effect of second winter feeding level and year (P<0.001), and management system (P=0.122). Ewe pre-mating weight was positively associated with litter birth weight. Litter birth weight for gimmers was significantly lighter than that for 3-8 years old ewes (P<0.01). Litters born to mature ewes or gimmers that received the standard feeding level in the first winter feeding phase were significantly heavier than those born to mature ewes or gimmers that received the corrective feeding level in the same period (P<0.001 or P<0.01, respectively).

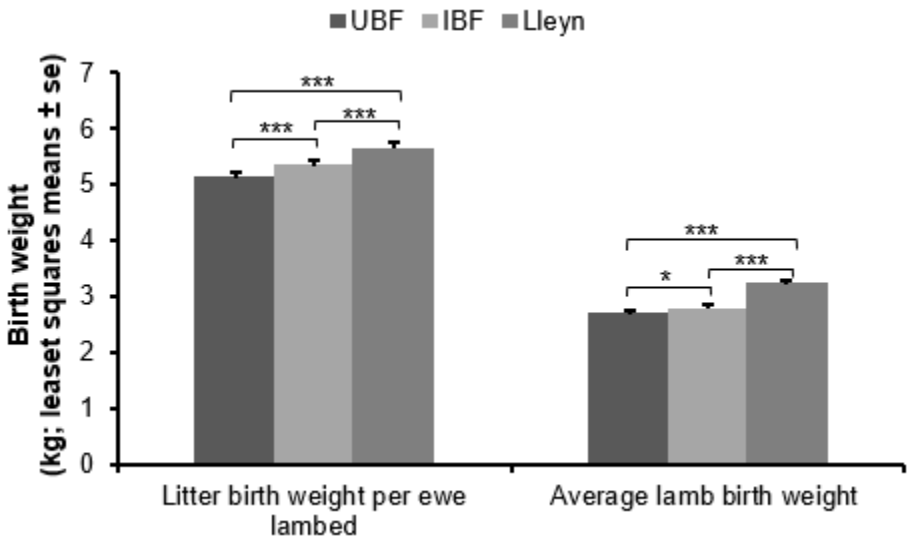


Figure 2-12. The litter birth weight and the average lamb birth weight (kg; least squares mean ± se) for the three genetic lines, when based on the ewes that gave birth to lamb(s).

The LMM analysis of average lamb birth weight (based on ewes that lambed) showed that Lleyn ewes achieved significantly heavier average lamb (i.e. offspring) birth weight than UBF and IBF ewes (P<0.001 and P<0.001, respectively; Figure 2-12), IBF ewes achieved significantly greater average lamb birth weight than UBF ewes (P<0.05), when adjusting for ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P=0.021), ewe age (P=0.289), first winter feeding level (P=0.657), interaction effect of second winter feeding level and year (P=0.002), litter sex (P<0.001) and management system (P=0.223). Ewe pre-mating weight was positively associated with average lamb birth weight. Among singletons, the average lamb birth weight of males was heavier than that of females (P<0.001; predicted means: male = 3.92 kg, female = 3.69 kg). Similarly, the average lamb birth weight of male-male twins was greater than that of female-female twins (P<0.05; predicted means: male-male twin = 3.20 kg, female-female twin = 3.05 kg). The average lamb birth weight did not differ significantly between male-male and male-female or male-female and female-female twin litters (predicted mean for male-female twin = 3.14 kg).

2.4.1.3 Weaning weight

As barren rates and litter sizes had been reported in the preceding sections, this section presents results as weaned litter weight per ewe that lambed and average lamb weaning weight per ewe that weaned lambs, for investigation of ewes' ability for rearing lambs. Among the ewes that gave birth to lambs, there was no significant difference in weaned litter weight per ewe that lambed among the three genetic lines (P=0.149; Figure 2-13), when the LMM model adjusted for ewe pre-mating weight (P<0.001), ewe age (P=0.005), first winter feeding level (P<0.001), interaction effect of second winter feeding level and year (P<0.001), lamb age at weaning (P<0.001) and management system (P=0.154). Ewe pre-mating weight and lamb age at weaning were positively associated with weaned litter weight. This parameter was significantly higher for mature ewes and for gimmers fed with the standard feeding level in the first winter feeding period than for their counterparts fed with the corrective feeding level in the same period (P<0.001 for mature ewes and for gimmers). Litter weights weaned from gimmers were lighter than those weaned from 3-6 years old ewes (P<0.01 for each pairwise Student's t-test). Litter weights weaned from 3 years old ewes were lighter than those weaned from 4-5 years old ewes (P<0.01 and P<0.05, respectively).

Average lamb weaning weight per ewe that weaned lambs did not differ among the three genotypes (P=0.087; predicted means: UBF = 27.46 kg, IBF = 27.61 kg, Lleyn = 28.01 kg; Figure 2-13), when ewe pre-mating weight (P<0.001), ewe age (P=0.396), first winter feeding level (P=0.704), interaction effect of second winter feeding level and year (P<0.001), lamb age at weaning (P<0.001), weaned litter sex (P<0.001) and management system (P=0.012; predicted means: CON = 27.97 kg, PLF = 27.45 kg) were adjusted for in the LMM model. Ewe pre-mating weight and lamb age at weaning were positively associated with average lamb weaning weight per ewe that weaned lambs. Male lambs weaned as singletons were heavier than their female counterparts (P<0.001; predicted means: male = 29.08 kg, female = 26.98 kg). Similarly, average lamb weaning weight per ewe that weaned lambs of male-male twin litters was significantly heavier than that of female-female twin litters (P<0.01; male-male twin = 28.30 kg, female-female twin = 26.91 kg). The average lamb weaning weight per ewe that weaned lambs did not differ significantly between

male-male and male-female twins or male-female and female-female twins (predicted mean for male-female twin = 27.51 kg).

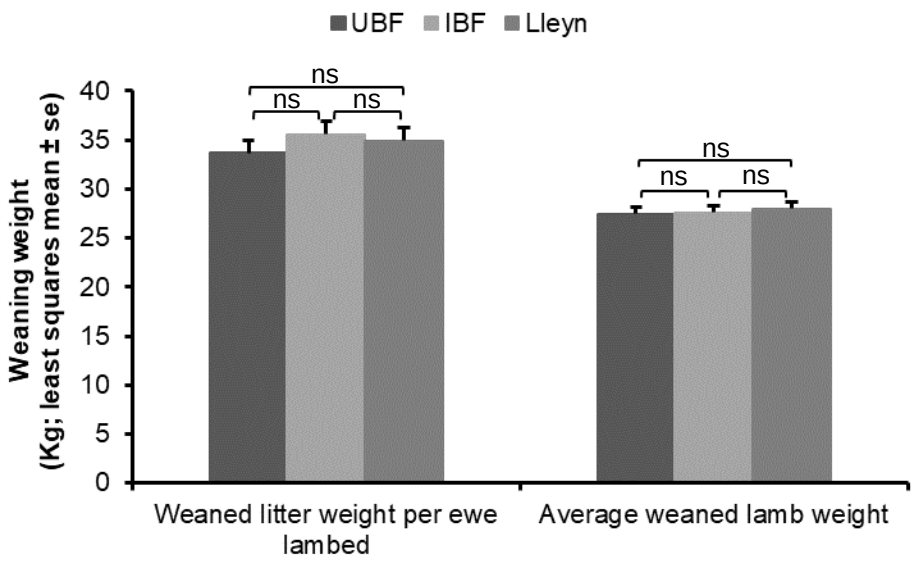


Figure 2-13. The weaned litter weight per ewe that lambed and the average lamb weaning weight per ewe that weaned lambs (kg; least squares mean ± se) for the three genetic lines.

2.4.1.4 Lamb growth rate

Between birth and marking, IBF and Lleyn lambs grew significantly faster than UBF lambs ($P<0.01$ and $P<0.05$, respectively; Figure 2-14) but lamb growth rate did not differ between IBF and Lleyn lambs, when LMM analysis accounted for lamb birth weight ($P<0.001$), lamb sex ($P<0.001$; predicted means: male = 239.6 g/day, female = 225.4 g/day), litter size at birth ($P<0.001$), lamb age at marking ($P=0.360$), dam age ($P<0.001$) and management system ($P=0.873$). Lamb growth rate between birth and marking was positively associated with lamb birth weight. Lambs born to 3-5 years old ewes grew significantly faster than those born to gimmers ($P<0.001$, $P<0.001$ and $P<0.001$ for lambs born to 3, 4 and 5 years old ewes versus those born to gimmers, respectively). Twins grew significantly quicker than singletons and triplets ($P<0.001$ and $P<0.001$, respectively; Figure 2-15) in this investigated period.

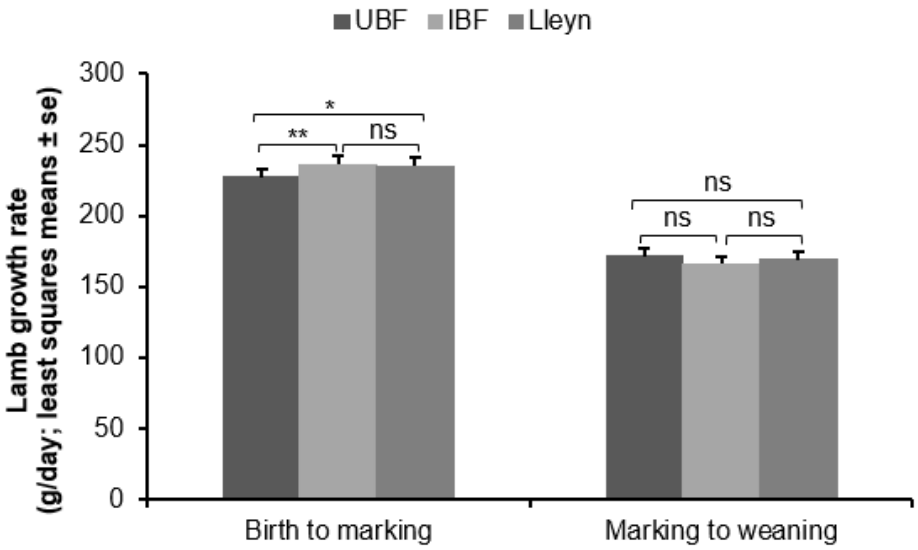


Figure 2-14. Lamb growth rate (g/day; least squares mean ± se) between birth and marking, and between marking and weaning, for the three genetic lines.

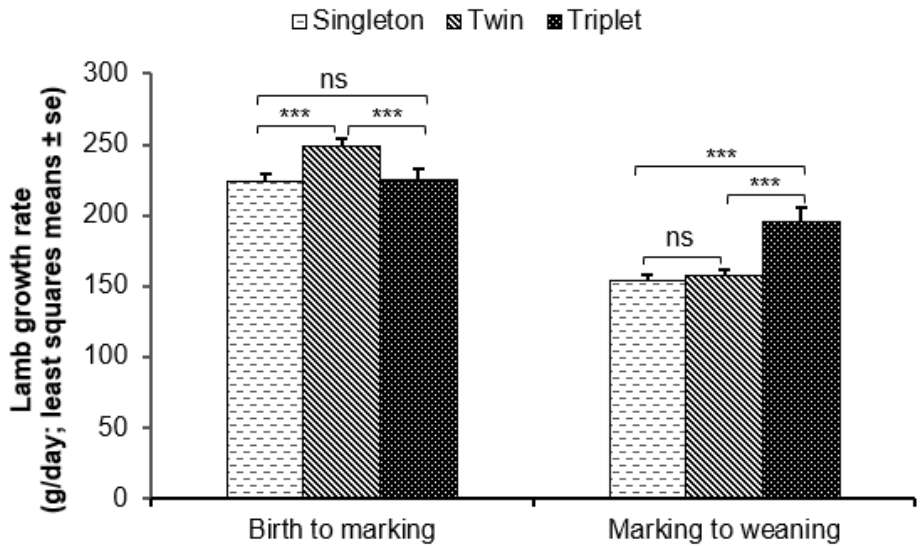


Figure 2-15. Lamb growth rate (g/day; least squares mean ± se) between birth and marking, and between marking and weaning, for singletons, twins and triplets.

Between marking and weaning, there was no significant difference in lamb growth rate among the three genetic lines (P=0.454; Figure 2-14), when adjusted for lamb sex (P<0.001; predicted means: male = 174.0 g/day, female = 163.8 g/day), litter size at weaning (P<0.001), number of days between marking and weaning (P=0.665), lamb age at marking (P<0.001), dam age (P=0.408) and management system (P=0.044; predicted means: CON = 171.4 g/day, PLF = 166.4 g/day). Lamb

age at marking was negatively associated with lamb growth rate between marking and weaning. Lambs weaned as triplets grew faster than those weaned as singletons and twins (P<0.001 and P<0.001, respectively; Figure 2-15) in this investigated period.

2.4.2 Pre-lambing metabolic profile (March 2015)

The adjusted means of BOHB, albumin, urea N, copper and magnesium concentrations for UBF, IBF and Lleyn twin-bearing ewes were all within the recommended ovine reference ranges (Table 2-9).

Table 2-9. Metabolite concentration values (least squares mean ± se) for twin-bearing ewes of the three genetic lines. Means in the same row with different superscript letters were different (P<0.05). Column 2 indicates published presumptive safe reference ranges.

Plasma metabolite	Reference range	UBF	IBF	Lleyn
BOHB (mmol/l)	<0.8[1]	0.43 ± 0.03[a]	0.43 ± 0.03[a]	0.41 ± 0.03[a]
Albumin (g/l)	25-35[2]	31.30 ± 0.42[a]	31.78 ± 0.44[a]	31.53 ± 0.42[a]
Urea N (mmol/l)	3-8[2]	3.41 ± 0.21[a]	3.33 ± 0.21[a]	3.9 ± 0.22[a]
Copper (µmol/l)	9.4-19.0[3]	13.3 ± 0.76[a]	13.53 ± 0.79[a]	13.52 ± 0.75[a]
Magnesium (mmol/l)	0.7-1.3[3]	0.89 ± 0.02[a]	0.90 ± 0.02[a]	0.99 ± 0.02[b]

[1]Sargison, (2008); [2] Kerr, (2002); [3] Dairy Herd Health and Productivity Service, (2001).

For BOHB concentrations, none of the variables, except the second winter feeding level (P=0.003), had a significant effect on plasma concentration of this metabolite (P>0.05). UBF ewes fed with the standard feeding level in the second winter feeding period had significantly higher BOHB concentrations than their counterparts fed with the corrective feeding level during the same period (P<0.01). There was no significant difference in the BOHB concentration between ewes receiving the standard feeding level and those receiving the corrective feeding level in the second winter feeding period for IBF and Lleyn ewes (P>0.05).

The concentration of albumin was positively associated with ewe BCS at scanning (P=0.039), but it was not significantly affected by genetic line (P=0.758), litter birth weight (P=0.166) or number of days between sampling and lambing (P=0.455). All the ewes (that lambed between 25 and 46 days after blood sampling; same applies for the rest of the pre-lambing metabolic profile results), except one IBF ewe (36 g/l), had an albumin plasma concentration within the reference range.

The status of urea N did not differ among genetic lines (P=0.156), nor on the basis of age (P=0.064) or management system (P=0.263), but it was positively associated with ewe BCS at scanning (P=0.028). Within each genotype, ewes fed with the corrective feeding level in the second winter feeding period had significantly higher urea N concentration than their counterparts fed with the standard feeding level (P<0.05, P<0.01 and P<0.05 for UBF, IBF and Lleyn ewes, respectively). Among all the ewes tested for this metabolite, 15 ewes (five UBF, six IBF and four Lleyn ewes) had urea N concentration beneath the lower threshold of the reference range, with a range from 1.5 to 2.7 mmol/l.

The copper concentration was not significantly influenced by any of the following: genetic line (P=0.972), ewe BCS at scanning (P=0.214), first winter feeding level (P=0.215), litter birth weight (P=0.229), number of days between sampling and lambing (P=0.249) or management system (P=0.319). Five ewes (three UBF and two Lleyn) had copper concentrations lower than the lower threshold of the reference range, while two UBF ewes had copper concentrations higher than the upper threshold of the reference range.

Lleyn ewes had significantly higher magnesium concentrations than UBF and IBF ewes (P<0.001 and P<0.01, respectively). The level of plasma magnesium did not differ significantly between UBF and IBF ewes (P>0.05). In the case of other model variables, ewe weight at scanning (P=0.103), litter birth weight (P=0.263) and number of days between sampling and lambing (P=0.174) did not have significant effects on the status of this metabolite. All the ewes, except one IBF ewe (0.64 mmol/l) had magnesium concentrations within the reference range.

2.4.3 Colostrum quality in the 2015 lambing season

The estimated mean SG values (± se) of 10 times diluted colostrum samples were 1.024 (± 0.0007), 1.024 (± 0.0007) and 1.022 (± 0.0006) for UBF, IBF and Lleyn twin-bearing ewes, respectively. Genetic line (P=0.406), ewe age (P=1.000), time between lambing and sampling (P=0.208) and litter birth weight (P=0.110) did not have significant impacts on SG values of colostrum samples.

2.4.4 Post mortem examination in the 2015 lambing season

In the Kirkton flock, the mortality rate of the pure-bred lambs was 12% (126 dead lambs out of 1048; 13% for UBF lambs, 13% for IBF lambs, and 10% for Lleyn lambs) in the 2015 lambing season. Of the 126 dead lambs, 29.4% were UBF lambs, 34.9% were IBF lambs, and 35.7% were Lleyn lambs.

Seventy-six lambs, comprising 16 UBF, 30 IBF and 30 Lleyn lambs (Table 2-10 and Figure 2-16), were examined *post mortem*. The results showed that 32 lambs died because of dystocia, 27 lambs died because of starvation/hypothermia, and 10 died of other known causes. For the other seven, findings were inconclusive.

Dystocia was the main primary cause of neonatal lamb death in the Kirkton flock. Among the examined lambs, 56% of UBF lambs, 43% of IBF lambs and 33% of Lleyn lambs had died as a consequence of dystocia. Lamb birth weight (P=0.008), but not genetic line (P=0.097), was a significant determinant for lamb death in the category of dystocia. Of the lambs examined, among the three genetic lines, UBF lambs had the lowest incidence of death from starvation/hypothermia, while Lleyn lambs had the highest incidence in this category. Within each genetic line, only Lleyn lambs that died of dystocia were significantly heavier than their counterparts which died of starvation/hypothermia (P<0.01).

Table 2-10. Distribution of lamb deaths in each 'cause of death' category by genetic line, gender and litter size.

		Dystocia	Starvation/ Hypothermia	Others	Inconclusive PM	Total
Genotype	**UBF**	9	4	3	0	16
	IBF	13	9	3	5	30
	Lleyn	10	14	4	2	30
Gender	**Male**	17	15	5	4	41
	Female	15	12	5	3	35
Litter size	**Single**	7	4	2	2	15
	Twin	22	33	6	4	55
	Triplet	3	0	2	1	6

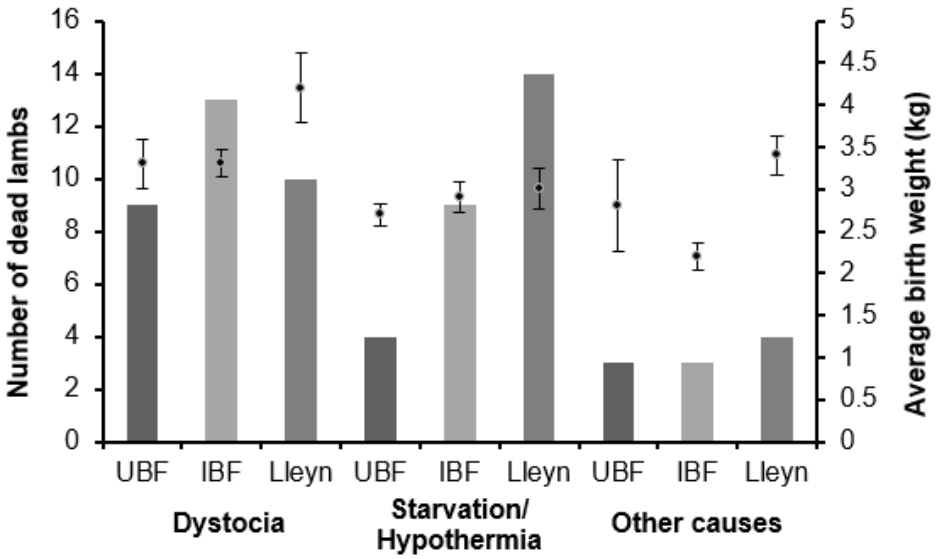


Figure 2-16. Number of dead lambs and average birth weights of the dead lambs, where cause was established, due to dystocia, starvation/hypothermia and other reasons for the three genetic lines. Bars indicate the numbers of dead lambs, and black dots show the mean dead lamb birth weight and vertical lines represent standard error of the mean.

2.4.5 Grazing behaviour observation (July 2015)

Raw data following observations of grazing ewes across seven days are presented in Figure 2-17 and Figure 2-18, for ewes observed within particular sectors and zones, respectively. UBF ewes grazed in all the accessible sectors, except sectors M and N; IBF ewes grazed in sectors A, C, F, G, H, I and M; Lleyn ewes preferred to graze in sectors C, D, F, H, I and L. Very low numbers of UBF and IBF ewes were observed in Middle west, Middle central and Middle east zones, and no Lleyn ewes were seen in those zones (Figure 2-18). UBF and IBF lambs were, for these observations, classified collectively as BF lambs, as they were not marked with different colours, so they could not be distinguished through direct observation. As shown in Figure 2-19 and Figure 2-20, the distributions of lambs followed similar patterns to those of their mothers.

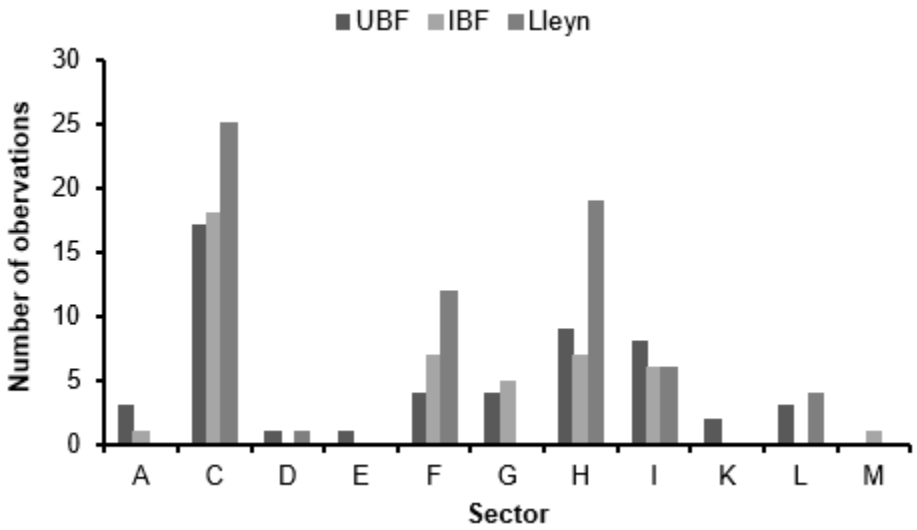


Figure 2-17. The number of observations of ewes from each genetic line in sectors A to M over the seven days of grazing observation. Sectors B and J were not accessible for sheep, while no ewes were observed in Sector N, thus these sectors were not indicated in this figure (same for Figure 2-19).

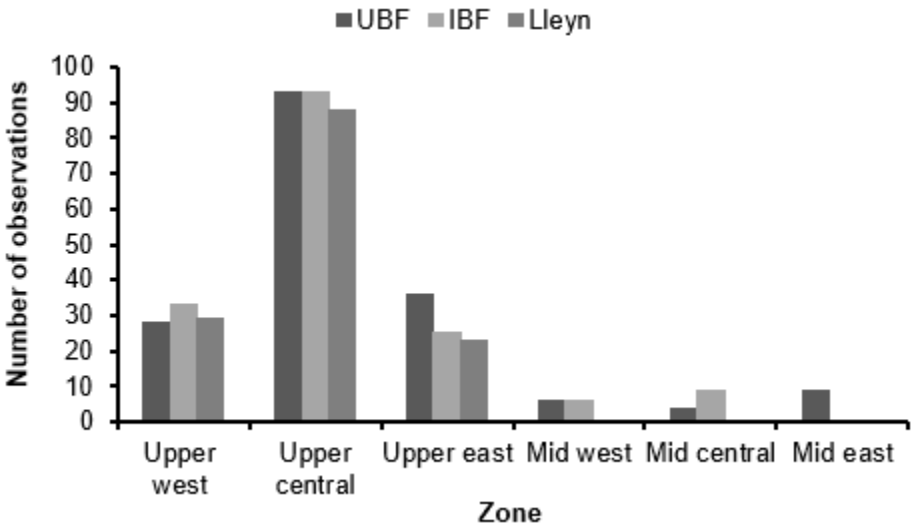


Figure 2-18. The number of observations of ewes from each genetic line in six distinct zones over the seven days of grazing observation.

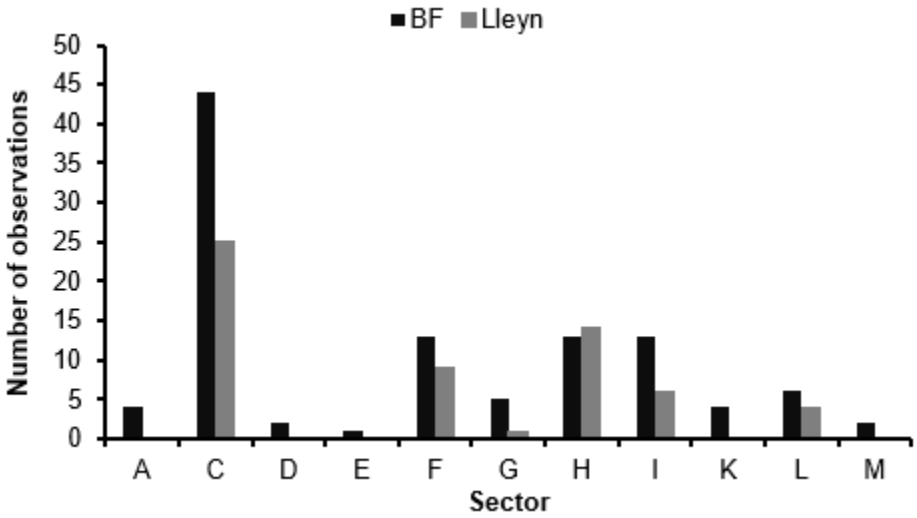


Figure 2-19. The number of observations of lambs from each breed in sectors A to M over the seven days of grazing observation.

GLMM analysis showed that number of ewes observed differed per sector (P=0.013), while there was no evidence suggesting that genetic line had significant impact on this response variate (P>0.05). More ewes (combining all three genetic lines) were observed in Sector C than in Sectors F and I (P<0.05 and P<0.05, respectively), and more ewes were observed in Sector H than in Sector F (P<0.05). There were more ewes in the upper central area during the seven days observation than in the upper west and the upper east areas (P<0.001 and P<0.001, respectively).

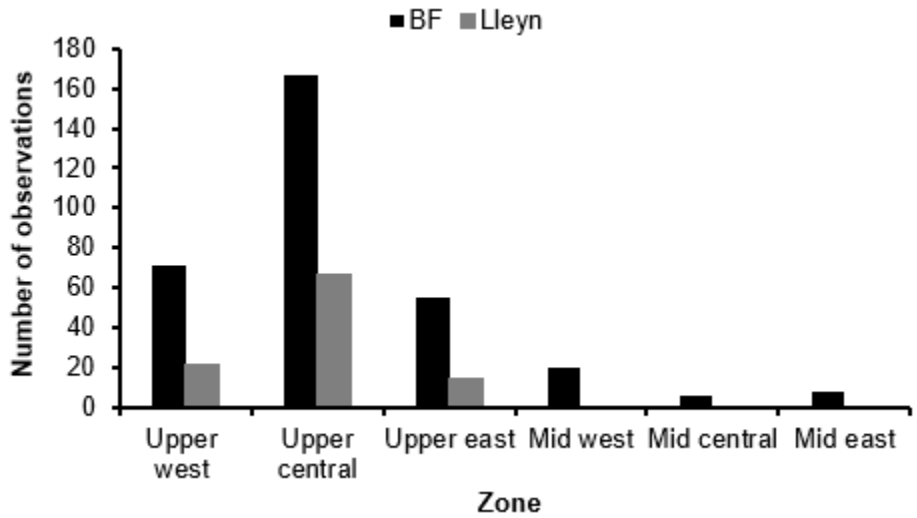
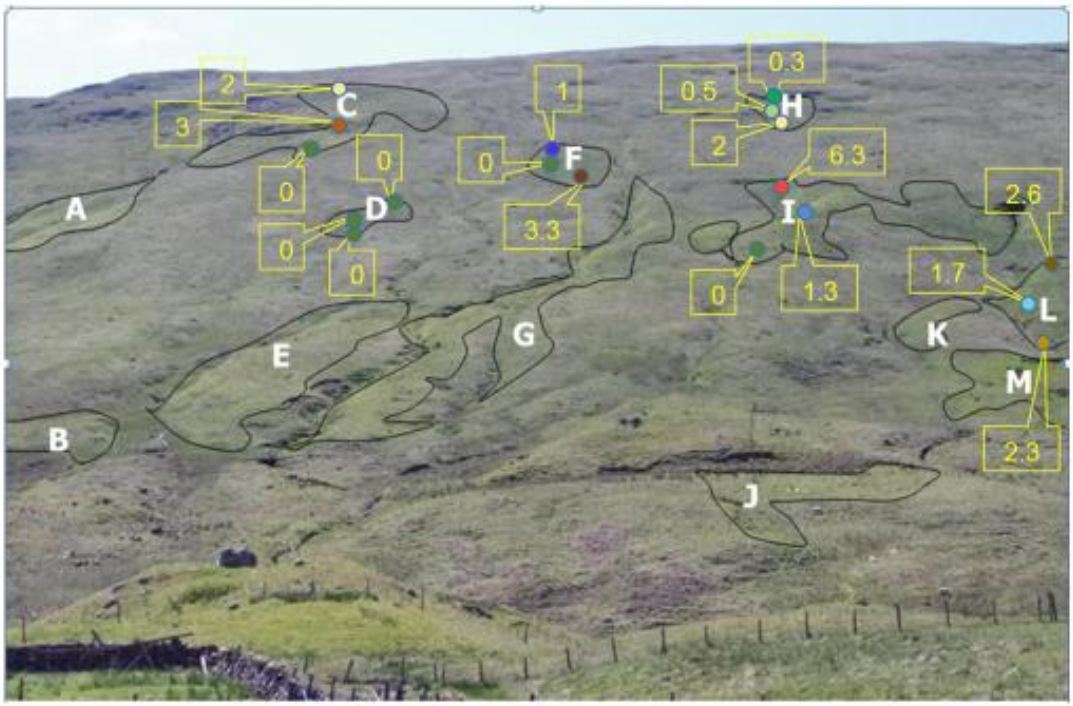


Figure 2-20. The number of observations of lambs from each breed in six distinct zones over the seven days of grazing observation.

In Sectors C, F, H, I and L, bog asphodel occurrence was recorded, and this revealed that densities differed (Figure 2-21). Sector D was the only sector where Lleyn ewes grazed that had no bog asphodel observed. In 2015, 18 lambs (four UBF, eight IBF and six Lleyn) were visibly affected by plochteach.



0	0.3	0.5	1	1.3	1.7	2	2.3	2.6	3	3.3	6.3
Green			Blue			Yellow			Amber		Red

Figure 2-21. The densities of bog asphodel in higher, middle and lower sampling quadrats of sectors C, D, F, H, I and L (number of plants/m^2), indicated in yellow digits. The original photograph of the hill site for grazing observation is shown in Appendix 2-2.

2.5 Discussion

This investigation over three production years showed that Lleyn ewes were as competent as their UBF and IBF flockmates in a Scottish hill environment. As a popular lowland/upland prolific sheep breed (Ceyhan et al., 2015), the reproductive performance of Lleyn ewes might be expected to be more adversely influenced by a harsh environment. However, these Lleyns achieved significantly greater litter sizes

at scanning, lambing and weaning than both the selected BF ewes (IBF) and the traditional hardy hill group – unselected BF ewes (UBF). The latter would be expected to have a litter size of 1.1-1.4 lambs born per ewe mated, when managed extensively in a hill environment (Dwyer & Lawrence, 2005). In the current study, UBF and IBF ewes had lower litter sizes (0.92 and 0.97 lambs per ewe, respectively) than that expectation. Only Lleyn ewes achieved a mean litter size of a similar magnitude (least squares mean = 1.15 lambs per ewe mated) as that expectation, and that was higher than reported for BF ewes (99 lambs per 100 ewes; Morgan-Davies et al., 2008a). Moreover, the significant limitation of extensive hill farming is that nutritional supply via grazing cannot meet the energy requirement of pregnant ewes (Robinson et al., 2002), which might result in lighter lambs with lower lamb vigour at birth (Robinson et al., 1999), and maternal undernutrition could also compromise the expression of maternal behaviour at lambing (Dwyer et al., 2003). Consequently, a large litter size with a higher proportion of multiple births would not be good for hill farming systems, especially when that is a factor influencing lamb survival (Knight et al., 1988; Knight, 1990).

Over the three years, Lleyn ewes had significantly lower barren rates than both UBF and IBF ewes, which suggested that the fertility (in terms of conception rate) of these Lleyns were not compromised when being farmed in the moderately extensive hill conditions. The higher barren rates at pregnancy scanning in 2014 (the preliminary analysis showed that barren rate of 2014 was significantly higher than those in 2013 and 2015; $P<0.001$ and $P<0.001$, respectively) could have been influenced by the high level of rainfall in December 2013 (651.2 mm), compared to that in December 2012 and 2014 (402.2 and 408.8 mm, respectively), during mating, as extreme weather conditions, such as being exposed to rain for six hours per day, could lead to a reduction in ovulation rate (Nichols, 1927; Griffiths et al., 1970).

It is well known that nutrition plays an important role in ewes' reproductive performance. Ewe body weight and BCS at mating reflect long-term nutritional status of ewes, and these parameters have positive associations with ovulation rates (Ducker & Boyd, 1977). The live weight reflects both body size and body condition (Ducker & Boyd, 1977), and that is positively associated with a ewe's BCS (Kenyon et al., 2004, 2014). BCS is a reliable and a widely accepted on farm tool to

assess ewe nutritional status (Phythian et al., 2012). For BF ewes, each point was found to represent 10.56 kg live weight in an early study (Russel et al., 1969). Furthermore, in ewes with moderately high BCS (up to 4; Robinson et al., 2002), body reserves would provide sufficient nutrients required for 'step by step' follicular development (Rassu et al., 2004). These ewes also have higher concentrations of follicle stimulating hormone (FSH; Rhind & McNeilly, 1986), the main regulator for ovulation rate, that promotes follicular growth if follicle diameter is greater than 2 mm (Rassu et al., 2004). A previous study of BF ewes showed that higher ewe BCS at mating (in the range of 1 to 3.5) increased ovulation rate and was associated with reduced embryo mortality (Gunn & Doney, 1975), and so increased lambing rate (Gunn et al., 1969). In this three year investigation, ewes were condition scored prior to mating season, and BCS ranged between 1.5 and 3.75. The discovery of the positive association between ewe pre-mating BCS and litter size at scanning and lambing was in agreement with Gunn et al. (1969) and Gunn & Doney (1975).

The purpose of the application of different winter-feeding levels was to provide minimal feed supplement for maintaining each ewe at reasonable body condition, in order to fulfil its reproductive potential. The proportions of ewes from each genetic line were similar in each feeding group. Additionally, any bias that might be caused by winter-feeding levels was taken out by including them into statistical analyses models. Thus, the comparison of ewe performance traits among the three genetic lines should be valid. In the current study, ewes that received the standard feeding level in the first winter feeding period were in better body condition at the start of that period, compared to their counterparts that received the corrective feeding level in the same period. Therefore, it is not surprising that mature ewes that received the standard feeding level had higher litter sizes at scanning and lambing (Gunn et al., 1969), and heavier litters at birth (Donald & Russell, 1970), compared to those that received the corrective feeding level. However, gimmers are still growing animals, and they have different priorities compared to adult ewes for partitioning nutrients during pregnancy, with more nutrients being used for maternal body growth and fat deposition (Wallace et al., 1996). This might explain why there were not significant differences in litter sizes at scanning, lambing and weaning, among the gimmers that received different feeding levels in the first winter feeding period.

The average birth weights of Lleyn and IBF ewes' lambs followed similar increasing trends over the three years. These may be expected due to the genetic selection indices used, where selection pressure is applied to increase lamb weights during growth (Conington et al., 2006). Lamb birth weight is a heritable trait, with an estimated heritability of 0.15 for both BF ewes (McLaren et al., 2012) and Lleyn ewes (Ceyhan et al., 2015). The average birth weights of Lleyn lambs in the 2014 and 2015 lambing seasons (Table 2-8) were heavier than found in a previous study of Lleyns recorded from different farms across the UK (mean ± SD: 3.89 ± 0.93 kg; Ceyhan et al., 2015). UBF and IBF ewes over the three years achieved similar or heavier average lamb birth weights as that reported previously for BF ewes (3.5 kg; Morgan-Davies et al., 2008a). Other factors, such as nutrition during pregnancy (Robinson et al., 1999) might be different between studies and vary between years, and thereby could contribute to the difference. Additionally, the birth weights of ewes themselves, which were not adjusted for in the corresponding statistical analysis model, might have a positive effect on their offspring's birth weight (Bradford et al., 1974). This could be considered as a variable for future investigation.

In hill sheep farming, lamb birth weight is a significant contributor to lamb survival (Fogarty et al., 2000). The U-shaped relationship between lamb birth weight and lamb mortality rate reported by Sawalha et al. (2007) and Dwyer (2008a) indicates that lambs with birth weight between 3.0 and 5.0 kg would have lower mortality rate, compared to lighter or heavier lambs. In the current study, the majority of the lambs (74%) had birth weights within the range (3.0-5.0 kg for BF lamb) reported by Dwyer et al. (2016), and consequently, the lamb mortality rates for all three genetic lines in the 2015 lambing season (13% for UBF, 13% for IBF and 10% for Lleyn) were lower than that reported from another Scottish hill flock (18% for BF; Morgan-Davies et al., 2008a).

Based on the results of post mortem examinations, dystocia was the main cause of neonatal lamb death in the flock in the 2015 lambing season. The relatively low starvation/hypothermia-related death rate might be because of some relatively good weather between April and May in 2015. Moreover, the flock was very closely managed in in-bye and semi-improved pasture (lower altitude areas) during lambing, which reduced the likelihood of new-born lambs dying from starvation or hypothermia. It is noteworthy that seven Lleyn lambs died within two days when

weather conditions were extremely bad, and six of them died of starvation/hypothermia, while among the 27 lambs that died of starvation/hypothermia, only four were UBF lambs. This scenario suggests that there is variation in resistance to cold exposure in neonatal lambs between the breeds and among the genotypes (Slee et al., 1980).

In the 2015 lambing season, most lambs that died of starvation/hypothermia were twins. One reason may be that individual twin lambs received less maternal care than their singleton counterparts (O'Connor et al., 1992). Additionally, twin lambs had lower birth weights than singletons. Light lambs not only have less brown adipose tissue (1-2% of lamb birth weight) that is used to generate heat via non-shivering thermogenesis in neonatal lambs (Symonds & Lomax, 1992), they also have lower plasma tri-iodothyronine and thyroxine at birth than heavy lambs of the same breed (Dauncey, 1990). Tri-iodothyronine is the thermoregulator for heat production from brown adipose tissue in neonatal lambs (Dauncey, 1990). In the foetal lambs, brown adipose tissue is rapidly accumulated from days 70 to 120 of gestation, and then accumulation slows down to term (day 147; Alexander, 1978). One Australian study of Border-Leicester x Merino ewes showed that maternal undernutrition (70% of energy requirements) between day eight of gestation and term exerted a moderate restriction on foetal growth, but doubled the weight of perirenal adipose tissue in their foetuses, compared to those carried by ewes being fed with 100% of total energy requirements (Budge et al., 2004). The increased deposition of brown adipose tissue would enhance heat production in those neonatal lambs. Furthermore, low birth weight lambs have a high ratio of body surface area to body mass, which accelerates the process of heat loss (Dwyer, 2008b). Moreover, the vigour of neonatal lambs is highly dependent on lamb birth weight (Sawalha et al., 2007); light lambs stand up slowly, and take more time to suck from mother than heavy lambs (Dwyer & Morgan, 2006). Of course, as previously described, maternal undernutrition compromises the expression of maternal behaviour and retards the establishment of ewe-lamb bonding at birth (Dwyer et al., 2003). This could also have an effect on lamb survival. On the other hand, heavy lambs are susceptible to dystocia, thus heavy singletons often have higher dystocia-related death rate than twins and triplets (Scales et al., 1986). In the 2015 lambing season, on the basis of the examined lambs, a higher percentage of twins than of singletons died as a consequence of dystocia (6% *vs.* 3%,

respectively). Additionally, 23 out of 32 lambs that died as a consequence of dystocia had birth weights less than or equal to 3.7 kg, below the 4 kg weight threshold considered critical by others (Sawalha et al., 2007; Speijers et al., 2010). Therefore, these deaths might have been because the size of the pelvis of those ewes was not sufficiently proportionate to the size of their lambs (McSporran & Fielden, 1979), or might have been due to malpresentation at birth, which occurs more frequently in lambs born in multiple litters (Dwyer & Bünger, 2012).

Lamb growth rate is an important indicator for success of sheep production enterprises (Ceyhan et al., 2015). This investigation showed that IBF lambs grew more quickly than UBF but not Lleyn lambs, in the early postnatal stage (birth to marking). In the first 56 days post lambing, 83% of lamb growth relies on the mother's milk production (Wallace, 1948; Snowder & Glimp, 1991; Treacher & Caja, 2002), which is influenced by maternal nutrition (Peart, 1968), ewe genotype (Peart et al., 1979) and the genotype of the sucking lamb (Peart et al., 1975). In the context of the current experiment, late pregnancy nutrition probably was not a significant cause of the presumptive variation in milk secretion among the three genetic lines, in twin-bearing ewes at least, as the plasma concentrations of BOHB, albumin and urea N in a cohort of twin-bearing ewes did not differ significantly among the three ewe groups. In the current study, only twin-bearing ewes were assessed for pre-lambing metabolic profiling, as they were under greater metabolic demands, but they were farmed on in-bye fields pre-lambing. Therefore, these cannot be extrapolated for single-bearing ewes grazing on the harsher hill ground, where genetic line differences may be tested more. Consequently, the higher growth rate of IBF lambs might suggest that IBF ewes have greater capability to produce better or more milk in the harsh hill environment. This outcome might also be influenced by other advantages of the IBF genetic line, such as heavier mature weight they own which is highly correlated with average daily gain of their lambs (Annett et al., 2011b,c), or better maternal care provided by IBF ewes that led them to establish a good ewe-lamb bond with their lambs. Alternatively, it could be explained by the genetic selection for lamb weaning weight in IBF lambs, which is a major contributor to the selection index, and would have had an impact on lamb growth rate (Yates & Pattie, 1970; Conington et al., 2006). Ultimately, IBF ewes achieved average lamb weaning weights per ewe that weaned lambs comparable to their Lleyn

counterparts, despite the fact that they had significantly lighter average lamb birth weights.

Between birth and marking, the lamb growth rate in all three genetic lines was lower than that of BF lambs reported by Speijers et al. (2010; 243 g/day). This could be a result of combined effects of genotype, nutrition and environmental conditions. Twins also grew faster than singletons during this period. This might be mainly because ewes that gave birth to twins were farmed in the areas where grass qualities and availabilities were better, compared to their counterparts that gave birth to singletons. That would potentially enhance the milk production for those ewes that had twins (Doney et al., 1981a). Moreover, in part, this might be influenced by compensatory growth (Wilson & Osbourn, 1960). Compared with single-bearing ewes, twin-bearing ewes probably suffered more from mild to moderate undernutrition during late pregnancy, although (as noted in Table 2-9) the pre-lambing metabolic indicators for energy and protein status were within the reference ranges (which indicate that any shortfalls were not extreme), at least in 2015. Of course, retardation of foetal growth can be caused by placental insufficiency during pregnancy, and could lead to reduced lamb birth weight and increased postnatal growth rate (De Blasio et al., 2007). One study suggested that the transition from foetal growth restriction to accelerated growth in early postnatal life is associated with increased abundance of insulin receptor in the skeletal muscle of nutrient restricted foetuses (Muhlhausler et al., 2009). This persists when nutritional supply becomes unrestricted in the early postnatal stage. These insulin receptors combine with insulin signalling molecules (i.e. phosphoinositide 3-kinase p85α subunit and protein kinases) and glucose transporter type 4 protein to promote lamb growth (Muhlhausler et al., 2009). In addition, among the lambs assessed for lamb growth rate in the current study, male lambs grew quicker than female lambs between birth and weaning, which was in agreement with Bermejo et al. (2010) and Bianchi et al. (2016). Such higher growth rates in the male lambs might be partly due to their heavier birth weight, which has been shown to have a positive influence on postnatal growth in lambs (Greenwood et al., 1998).

Ewes of IBF genetic line has been selected on a multi-trait selection index to progressively improve ewe and lamb performance, while UBF ewes has been selected for remaining close to the average performance of the flock before genetic

selection started in the flock in 1998 (Conington et al., 2006). Therefore, the litter birth weight per ewe that lambed and average lamb birth weight of IBF ewes were heavier than those of UBF ewes. However, the average weaned lamb weight and the weaned litter weight per ewe that lambed of IBF ewes were comparable to those of UBF ewes. This could be mainly due to the fact that the average lamb weaning weight of IBF ewes in 2015 was lighter than that of UBF counterparts (see Appendix 2-3). Thus, attention should be made on Signet selection index (Hill 2 Index) of IBF ewes, which might not select much divergence in lamb weaning weight between UBF and IBF ewes. Additionally, the rams of unimproved BF line bought in might had low accuracies in EBVs, that changed the selection index value of the UBF ewes, hence caution should be made for selecting rams for the following studies.

The two management systems, which had different criteria for worming, winter feeding and culling, did not affect most of the reproductive traits investigated: i.e. litter sizes at scanning, lambing and weaning; barren rate; litter birth weight; lamb birth weight; weaned litter weight per ewe that lambed. The PLF system reduced the expenditures for labour and usage of anthelmintics, which in turn would improve farm profitability (Morgan-Davies et al., 2018). Additionally, in the current experiment, ewe age was not a significant determinant for litter size at lambing nor for average lamb birth weight. These findings differ from the results reported by Annett et al. (2011) for 2 to 6 years old ewes (BF, Swaledale x BF, North Country Cheviot x BF, Lleyn x BF and Texel x BF) from six hill farms in Northern Ireland. The results concerning age effect on litter size at scanning and weaning, and weaned litter weight per ewe that lambed were similar to those reported by Annett et al. (2011). Ewes that were 6 years old or over, were all managed under the PLF system in this experiment. Consequently, ewes were culled according to their individual fitness, but not their age (Morgan-Davies et al., 2014, 2018), in that management system, whereas in the CON management system, ewes were culled after having had the fourth crop. This might have influenced the age effects on ewes' reproductive performance.

The nutritional requirements of ewes differ at different stages during the reproductive cycle, and vary according to the number of foetuses they are carrying (McDonald et al., 2011). In this experiment, pre-lambing metabolic profiles were used to determine the nutritional and metabolic status of late pregnant twin-bearing

ewes (O'Doherty & Crosby, 1998; Antunovic et al., 2011). These should be ideally conducted 2-3 weeks before lambing, when ewes are under high metabolic demand, but also, if required, there is time to adjust feeding rations based on the results (Russel, 1991; Dairy Herd Health and Productivity Service, 2014). Due to the constraints of the hill sheep farming system, ewes were blood-sampled 23-56 days before their lambing dates, but only the data from ewes that lambed within 25-46 days post-sampling were analysed, in order to provide results from within a relatively comparable time scale.

When ewes are in negative energy balance, they mobilise body fat reserves. BOHB is therefore generated to meet the energy requirement (Sargison, 2007; Dairy Herd Health and Productivity Service, 2014). The adjusted mean BOHB concentrations of UBF, IBF and Lleyn twin-bearing ewes were comfortably within the safe range (<0.8 mmol/l for adequately nourished ewes), and were lower than those of twin-bearing ewes (Booroola Merino x Poll Dorset, Trangie Fertility Merino x Poll Dorset, and Border Leicester x Merino) on two different feeding regimens (high and low) on day 112 of pregnancy (Fogarty et al., 1992). These outcomes were also lower than those of twin-bearing ewes (Suffolk cross) with six different feeding regimens on day 121 of pregnancy (O'Doherty & Crosby, 1998), which suggests that the twin-bearing ewes in the current study flock were adequately nourished with no early signs of pregnancy toxaemia at the blood sampling timepoint. The significant effect of feeding level on BOHB concentrations in the UBF ewes was in agreement with Fogarty et al. (1992). However, the mean BOHB value among UBF ewes that were fed with the standard feeding level in the second winter feeding period was still within the recommended range. Fogarty et al. (1992) also reported a significant effect of ewe genotype on BOHB concentration, which indicates that different genotypes of ewes have slightly different physiological mechanisms governing body fat mobilisation. This was not found to be the case in the current study.

Concentration of albumin has been used to monitor protein status in sheep. Low albumin concentration suggests long-term low protein intake, liver damage or blood loss (Dairy Herd Health and Productivity Service, 2014; Robinson, 2018). Rapid reduction in plasma concentrations of albumin can occur if protein content of feeds is low during late pregnancy (O'Doherty & Crosby, 1998). Concentration of urea N reflects the level of current protein intake (Caldeira et al., 2007; Dairy Herd Health

and Productivity Service, 2014; Robinson, 2018). This explains why ewes receiving the corrective feeding level in the second winter feeding period had higher urea N concentrations than their counterparts receiving the standard feeding level in the same period. The adjusted mean albumin and urea N concentrations for UBF, IBF and Lleyn twin-bearing ewes were within the recommended ranges and were higher than those of Suffolk cross twin-bearing ewes at day 121 of pregnancy in a study reported by O'Doherty & Crosby (1998). In contrast to the findings of Alonso et al. (1997; Merino, from ewe lambs to over 4.5 years old ewes), the concentration of albumin was not significantly affected by ewe age in the current experiment, albeit blood-sampled ewes were all aged 3-5 years old. Notably, 15 ewes (five UBF, six IBF and four Lleyn) had lower urea N concentrations than the lower threshold of the reference range, a status which could compromise foetal growth and udder development, and thus reduce production of colostrum (Robinson, 1985; Mellor & Murray, 1985; Robinson, 2018). These might affect lamb health and survival. However, further investigation was not conducted, as so few ewes had lower urea N concentrations in the current study.

Copper is an essential mineral element required for many enzymes' activity. Copper deficiency has a negative impact on ewe fertility, and can lead to abortion (Suttle, 2010a). In late pregnancy, low maternal copper status also has detrimental effects on development of the foetal nervous system, and can result in 'Swayback' in the lamb (Suttle & Jones, 2007; Dairy Herd Health and Productivity Service, 2014). Conversely, copper can be accumulated in the body and if its levels exceed the capacity of the liver to store copper, will cause chronic copper toxicity in sheep, with breeds such as Texel very susceptible (Suttle, 2010a). In the current experiment, the adjusted mean copper concentrations for the three genetic lines were within the reference range (Dairy Herd Health and Productivity Service, 2001), which indicated that there was no evidence of either copper deficiency or copper toxicity in the Kirkton flock.

Magnesium has catalytic, electrochemical and structural functions in animals (Suttle, 2010b). There are no body reserves of magnesium in sheep. Magnesium plasma concentration therefore indicates its level in the diet. Low concentrations of magnesium not only lead to 'Staggers' and hypocalcaemia in sheep (Suttle, 2010b; Dairy Herd Health and Productivity Service, 2014), but also compromise milk

production (Teh et al., 1985), ewe fertility and lamb growth (Gabryszuk & Klewiec, 1997). The adjusted mean magnesium concentrations for UBF, IBF and Lleyn twin-bearing ewes were within the reference range, and were similar to the level (0.94 mmol/L) reported by Alonso et al. (1997) for Merino ewes. Remarkably, Lleyn twin-bearing ewes had significantly higher magnesium concentrations than UBF and IBF ewes, a notable finding given that all the ewes were farmed together as flockmates. The significantly higher magnesium concentrations among Lleyn twin-bearing ewes might suggest that those ewes had higher dry matter intakes, at least during late pregnancy, thereby better providing sufficient nutrients to meet the requirements for their own maintenance and to support foetal growth, ultimately enabling them to produce heavier litters at lambing.

Twin-bearing ewes' colostrum qualities were determined using refractometer, expressed as SG values. This parameter did not differ significantly among the three genetic lines. SG value reflects refractive index of the colostrum sample (Mettler Toledo, 2014), which has good correlations with the concentrations of total protein and immunoglobulin determined using a zinc sulphate turbidity test, in sheep colostrum (correlation coefficients were 0.98 and 0.78, respectively; Harker, 1978). The current results, suggesting no difference in SG value among the three genetic lines, agree with results reported by Dwyer & Morgan (2006), when they investigated colostral IgG concentrations for BF and Suffolk ewes. In the current study, three out of 51 colostrum samples were measured following a single freeze-thaw cycle, however, the literature suggests that this should not have an effect on the accuracy of the measurement of SG value (Morrill et al., 2015). Nevertheless, SG reading can be affected by the temperature of the colostrum (Mechor et al., 1991). When these colostrum samples were analysed in the shed, neither temperatures of atmosphere nor of colostrum were determined. This could mean that the SG values were inaccurate. However, all the samples were measured in the same manner, so the comparison among the three genetic lines should be reasonable.

Grazing behaviour varies among breeds (Arnold et al., 1981; Dwyer & Lawrence, 1999), and might influence nutrient consumption in sheep, and subsequently affect their performance. The seven days of grazing observations showed that sector and zone of the hill grazing area had significant effects on the number of ewes observed

in these areas, as sheep have knowledge of their pasture and know where to find their preferred grass (Lawrence & Wood-Gush, 1988). The genetic line was not a determinant for number of ewes observed in the sector or zone, although Lleyn ewes preferred to graze on the areas (sector C, F and H) where the overall grass quality was better (the overall grass quality of top greener areas was better than those of other areas; Appendix 2-2; J Holland 2015, personal communication, July & August). The hill site used for grazing observation was a complex mosaic grassland, containing various vegetation species, including *Nardus stricta, Galium saxatile, Festuca ovina, Agrostis capillaris* and *Pteridium aquilinum* (Holland et al., 2008; J Holland 2015, personal communication, July & August). The actual type of grass that each animal grazed could not be determined by direct observation from the distance applied in the experiment. In future studies, the application of new technologies, such as the Internet of Things for studying grazing behaviour (Maroto-Molina et al., 2019), the next generation sequencing technology (Pompanon et al., 2012) and near-infrared reflectance spectroscopy (Akdağ & Ocak, 2019) for determining herbage intake would improve the accuracy of the study.

As a pilot study, this grazing behaviour observation had several other limitations. Firstly, ewes' fleece were dyed to different colours according to their genetic line (UBF: orange; IBF: brown; Lleyn: original white fleece), in order to distinguish them from other ewe genetic lines during observation, which might cause potential bias. Secondly, the inter-observer (two observers) reliability was not performed due to the low observation dates conducted in the experiment. Additionally, observations were only taken on the days of good visibilities, which might lead to bias, as weather condition could have impacts on sheep grazing behaviour (Mysterud et al., 2007b). Moreover, the grazing behaviour observed during the current observation cannot extrapolate to other times of the year, as ewes would be farmed in different locations (improved pastures, semi-improved pastures, or hill ground), according to their conditions and pregnancy status, and they would also be supplied with supplements during the winter.

The concern of farming two breeds together is that it might lead to competitive grazing relationship (Arnold & Pahl, 1974), and potentially increase the risk of exposure to toxic plants, such as bog asphodel. This plant is commonly believed to cause hepatogenous photosensitisation, referred to as plochteach in sheep. This is

a liver function-related disease, which normally affects 2- to 6-months-old lambs. The incidence of plochteach is up to 10% in many Scottish hill farms (Sargison, 2008), and the incidence varies between breeds, within breeds, and changes from year to year (Pollock et al., 2015). In the current flock, the incidence of the disease in 2015 (1.7%) was much lower than reported previously (Morgan-Davies et al., 2008b; Sargison, 2008), and was lower than that of the flock in 2014 (2.6%; Pollock et al., 2015). Observation of the density of bog asphodel in the sectors where ewes grazed frequently showed that this plant was present in most sectors, except sector D. The controversy about this plant is that the toxicity attributed to the plant in different regions might differ, although the proposed causative agents – saponins - remain at a similar level (Mysterud et al., 2007a), and the flower stems of the plant are more toxic than its leaves (Flåøyen et al., 1997). Pollock et al. (2015) suggested that consumption of bog asphodel alone might not cause plochteach, as previous direct feeding of a large volume of bog asphodel (20 g/kg lamb weight) did not induce the disease in Norwegian white lambs (Flåøyen et al., 1991).

2.6 Conclusion

The results over the three production years indicate that Lleyn ewes might have adapted well to the moderately harsh hill environment, and they may have the potential to improve the productivity and profitability of hill sheep farms, as the ewes of that breed had lower barren rate at ultrasound pregnancy scanning than UBF and IBF ewes, with significantly higher litter sizes at scanning, lambing and weaning than both BF genetic lines. Lleyn ewes also achieved heavier litter birth weights than UBF and IBF ewes. The litter weaning weights of Lleyns were heavier than those of UBFs, but were lighter than those of IBF flockmates. However, the differences were not significant among the three genetic lines. Dystocia was the primary cause of neonatal lamb death in the study flock. Lamb birth weight, but not the genetic line was a determinant for likelihood of lamb dying of dystocia. The grazing observation showed that sector had an effect on the number of ewes observed, but not the genetic line. Nevertheless, the resilience of Lleyns in a harsh hill environment could not be determined on the basis of just a three-year investigation, especially as none of the years included prolonged severe wintry conditions, so further investigation was recommended in the following two breeding seasons (as reported in Chapter 3).

Chapter 3: Investigation of reproductive performance of Lleyn and Scottish Blackface ewes managed under a moderately or a more extensive hill condition

3.1 Summary

The introduction of a lowland/upland prolific sheep breed, Lleyn, into hill sheep farming systems, or the genetic selection of an established hardy hill sheep breed, the Scottish Blackface (BF), could potentially improve productivity and so profitability of hill sheep enterprises (Chapter 2). Prior to providing recommendations to the industry, the reproductive performance of Lleyn ewes in a harsh hill environment was further investigated between November 2015 and October 2017. In this phase of the hill flock study, three genetic lines of ewes (approximately 200 UBF, 200 IBF and 200 Lleyn) were managed together either in a predominantly 'Hill Grazing' or 'Park Grazing' management system, each system having different criteria for using grazing resources and feed supplements. The results presented in this chapter were based on the data files of ewes mated with rams of their own genotype for two oestrous cycles, except for the barren rates, which also included the data from the third oestrous cycles, when ewes were mated with the rams from the opposite breed.

Averaging across the Hill and Park Grazing systems, the results of the two-year investigation (Nov 2015 to Oct 2017) showed that Lleyn ewes had comparable barren rates to their UBF and IBF flockmates. The litter sizes per ewe mated at scanning, lambing and weaning did not differ significantly among the three genetic lines ($P>0.05$). However, after removing barren ewes from the analyses, Lleyn ewes weaned more lamb(s) than UBF and IBF ewes ($P<0.01$ and $P<0.05$, respectively; weaned litter size per ewe lambed: 1.40 *vs.* 1.24 and 1.26, respectively). When based on all the ewes that lambed, Lleyn ewes had greater litter birth weights than UBF and IBF ewes ($P<0.001$ and $P<0.001$, respectively; 6.37 *vs*. 5.31 and 5.70 kg, respectively), and IBF ewes had heavier litter birth weight than UBF ewes ($P<0.01$). The average lamb birth weight of Lleyn ewes (averaged across the two systems) was significantly higher than those of UBF and IBF ewes ($P<0.001$ and $P<0.001$,

respectively; per ewe lambed: 3.75 *vs*. 3.28 and 3.47 kg, respectively). At weaning, Lleyn ewes had heavier litters than UBF and IBF ewes (P<0.001 and P<0.001, respectively; per ewe that lambed: 38.49 *vs*. 30.97 and 32.84 kg, respectively), whereas this parameter did not differ significantly between UBF and IBF ewes (P>0.05).

The relevant examinations conducted in Chapter 2 were continued in the investigation presented in this chapter. The pre-lambing metabolic profile showed that Lleyn twin-bearing ewes had significantly higher magnesium plasma concentrations than UBF and IBF ewes (P<0.001 and P<0.01, respectively), which suggested that Lleyns might have higher feed intake than their BF counterparts. In addition, they had significantly lower BOHB (P<0.05 and P<0.01, respectively) and significantly higher urea N than their BF counterparts (P<0.001 and P<0.001, respectively). IBF twin-bearing ewes produced better quality colostrum than their UBF counterparts (P<0.01), but not their Lleyn counterparts (P>0.05), based on Brix refractometer results. In the 2016 and 2017 lambing seasons, lamb mortality rates for UBF, IBF and Lleyn lambs were 7 and 5%, 14 and 8%, and 9 and 4%, respectively. The post mortem examinations revealed that dystocia remained the main cause of neonatal lamb death in the Kirkton flock (58 out of 92 examined lambs). However, lambing difficulty (assisted or non-assisted lambing) was not significantly influenced by ewes' external pelvic width (P=0.979). Overall, Lleyn ewes performed as competently as UBF and IBF ewes in terms of litter size at lambing and weaning in a harsh hill condition; they were also able to produce heavier litters at lambing and yield heavier litters at weaning then their BF counterparts.

3.2 Introduction

The preliminary investigation of ewe reproductive performance among the UBF, IBF and Lleyn ewes mated with rams of their own genetic line was conducted between November 2012 and October 2015, to compare the effect of breed improvement and breed substitution on improving productivity of hill sheep enterprises. The results showed that Lleyn ewes had lower barren rate and higher litter sizes per ewe mated at weaning than both BF genetic lines, with comparable weaned litter weight per ewe that lambed to those of UBF and IBF ewes, thus Lleyn may be a suitable breed

for improving output and profitability of hill sheep enterprises (Chapter 2; Zhou et al., 2017). However, the weather conditions were generally mild in those three winters (World Weather Online, 2018) and use of the higher hill areas of the farm was limited during some periods of the year, which may not reflect hill sheep management systems on farms where in-bye land is more scarce. A further investigation of ewes' reproductive performance among the three ewe groups (UBF, IBF and Lleyn) was therefore performed in the following two ewe reproductive cycles, with two contrasting management systems (Hill Grazing *vs.* Park Grazing; detailed in Materials and Methods section).

Extensive hill sheep farming utilises hill and montane areas. These rough grazing lands support a range of vegetation types with different nutritive values, and have different species composition and productivity (Armstrong et al., 1997; Holland et al., 2008). The SRUC Hill & Mountain Research Centre comprises improved pasture (flat in-bye fields; 170 metres in altitude), semi-improved pasture (~ 300 metres in altitude) and hill ground (300 to 1,025 metres in altitude). The productivity, digestibility, palatability and nutritive value of the vegetation differs among these land categories. The latter two are composed of a mosaic of grassland and mire communities, where the dominant grass species are *Nardus stricta, Festuca vivipara* and *Agrostis capillaris* (Holland, 2001). *Nardus stricta* is one of the dominant species in the rough grazing lands in the north-west of the UK. *Nardus stricta*-dominated grasslands are low in grazing value (Smith, 1918). The prediction model of vegetation biomass developed for grazing lands in the hill areas of the UK suggested that altitude had an impact on dry matter production in grassland (Armstrong et al., 1997). In the *Nardus*-dominated grassland, only *Agrostis-Festuca* is grazed by sheep. The annual dry matter production of *Agrostis-Festuca* as reported to decline by 428 kg ha^{-1} (42.8 g m^{-2}) for every 100 metres increment in altitude, within an altitude range of 75 to 825 metres (Armstrong et al., 1997).

The two management systems applied in the current study provided an opportunity to examine ewe performance in different environmental conditions, where grass qualities (therefore nutritional supply) for grazing and meteorological conditions differed. Stressors, such as nutritional restrictions and harsh environmental situations can activate HPA (hypothalamic-pituitary-adrenal) axis activity, that in turn has negative impacts on HPG axis (hypothalamic-pituitary-gonadal, demonstrated in

Chapter 1; see Section 1.7.1 and 1.7.2) and depresses ewe reproductive performance (Dobson et al., 2012; Narayan & Parisella, 2017). Undernutrition can compromise ewe reproductive performance by reducing ovulation rate, increasing embryonic mortality, reducing lamb birth weight and lamb vigour, and exerting negative impacts on offspring reproductive performance (Russel, 1991; Nottle et al., 1997; Robinson et al., 1999, 2002; Sen et al., 2013). Extreme weather conditions also have various effects on ewe reproductive performance. Several studies demonstrated that application of artificial rainfall (6 h/day; provoking the effect of cold, Webster & Park, 1967) prior to or during mating could result in lowered ovulation rate, increased embryonic mortality and delayed onset of oestrous cyclicity (Nichols, 1927; Griffiths et al., 1970; Doney et al., 1973, 1976). Another experiment revealed that cold stress could depress oestrus in shorn or fasting ewes (MacKenzie et al., 1975). However, well adapted adult ruminants with a good feeding regimen and full fleece would be very hardy in dry and cold conditions (Young, 1983).

Extreme weather conditions, such as cold and wet weather in the Scottish hill environment might be challenging for lamb survival. Cold exposure has been suggested as being a major factor adversely affecting lamb viability, teat-seeking activity and survival (Alexander & Williams, 1966; Alexander, 1973; Slee & Stott, 1986). This might apply to Lleyn lambs, being traditionally adapted as a lowland/upland sheep breed, when born in a harsh hill environment (Chapter 2), as their skin thickness and cold resistance might not be as strong as those of BF lambs for coping with harsh weather to the extent necessary to survive in such conditions (Samson & Slee, 1981). These limitations might affect the survivability of Lleyn lambs. Another researcher reported that exposing pregnant ewes to cold conditions during mid and late pregnancy could improve the cold resistance of their lambs (Labeur, 2018). The survivability of pure-bred Lleyn lambs born from ewes farmed in this hill environment is therefore worth investigating.

Based on the post mortem examination results of the 2015 lambing season (Chapter 2), dystocia was the main cause of neonatal lamb death in the Kirkton flock, with 32 out of 76 examined lambs having died as a consequence of this. Dystocia was also the most frequent cause of neonatal lamb death for UBF (9 out of 16 examined lambs) and IBF (13 out of 30 examined lambs) lambs. Lambs dying as a consequence of dystocia has been reported to account for 9% to more than 50% of

neonatal lamb deaths (Brown et al., 2014; Refshauge et al., 2016). It is mainly due to the size of the maternal pelvis being disproportional to the size of the lamb (McSporran & Fielden, 1979). Ewes' pelvises can be non-invasively measured externally using a calliper (Bassett, 1955; Adam, 2014) that enables a preliminary assessment of the association between presumptive pelvic dimension and incidence of dystocia (Quinlivan, 1971; Fogarty & Thompson, 1974).

This chapter examined the hypothesis that Lleyn ewes would have outperformed/equalled the performance of UBF and IBF ewes, with higher litter size, heavier lamb birth weight and heavier lamb weaning weight, when being managed under the Hill Grazing (more extensive) and the Park Grazing (moderately extensive which was similar to the system in Chapter 2) management systems in a Scottish hill environment. The objectives of the investigation were: *1),* characterising ewe reproductive performance (e.g. litter size, litter/lamb birth weight, litter/lamb weaning weight) and lamb growth rate among the three sheep groups; *2),* examining nutritional status of twin-bearing ewes in late pregnancy via metabolic profiling; *3),* assessing colostrum quality of twin-bearing ewes among the three genetic lines; *4),* determining primary causes of neonatal lamb death in the flock, and examining any breed differences in the different death categories; *5),* comparing ewe external pelvic width among the three genetic lines, and determining any correlation between ewe external pelvic width and lambing difficulty.

3.3 Materials and Methods

The investigation of ewe reproductive performance between November 2015 and October 2017 was conducted using the Kirkton flock at the SRUC Hill & Mountain Research Centre. The study site and the genetic selection of the ewes have been detailed in Chapter 2, Sections 2.3.1 and 2.3.2, respectively.

In November 2015, before ewes were joined with rams for mating, the flock size was reduced from approximately 300 ewes per genetic line to approximately 200 ewes per genetic line. At mating in November 2015, ewes were equally assigned to either a 'Hill Grazing' or 'Park Grazing' management system (Appendix 3-1), based on genetic line, ewe age, body weight, litter size and sire family. Each system had different criteria for using grazing resources and feed supplements. Both systems

were subject to the PLF approach previously developed (Morgan-Davies et al., 2014, 2018) and discussed and applied in Chapter 2.

The mating was managed in the same way as detailed in Section 2.3.2. Ewes were ultrasound pregnancy-scanned in mid-pregnancy (22nd/23rd and 20th/27th February for 2016 and 2017, respectively). Winter supplementary feeding levels (standard and corrective; see Table 2-2) were supplied in the same manner as described in Section 2.3.2 (Wishart et al., 2015). The first winter feeding phase was during mid-pregnancy from early January (6th/7th and 9th/10th January for 2016 and 2017, respectively) to scanning, while the second winter feeding phase was during late pregnancy from scanning to lambing, which started in mid-April (15th and 17th April for 2016 and 2017, respectively; see Figure 3-1 and Figure 3-2). In both figures, good single and good twin refer to single-bearing and twin-bearing ewes that maintained or put on weight since pre-mating, respectively; poor single and poor twin refer to single-bearing and twin-bearing ewes that lost weight since pre-mating, respectively; while triplet refers to triplet-bearing ewes. The decision on which feeding level each ewe would receive in each phase was made based on an individual ewe's live weight, BCS and pregnancy diagnosis (Table 2-2).

During lambing (Figure 3-1 and Figure 3-2), single-bearing ewes were brought into the improved pasture in-bye fields. Twin-bearing ewes were kept on the improved pastures during the daytime and were brought into the shed overnight. Triplet-bearing ewes were kept in the shed or on the improved pasture for lambing.

After lambing (Figure 3-1 and Figure 3-2), ewes managed under the Hill Grazing system were moved to the semi-improved pastures then into the hill ground with their lambs, whereas ewes managed under the Park Grazing management system were retained in the semi-improved pastures with their lambs. From the end of June, ewes managed under the Park Grazing system that gave birth to female singletons were farmed on the hill ground with their lambs, whereas their counterparts that gave birth to male singletons were retained in the semi-improved pastures with their lambs. Ewes managed under the Hill Grazing system that gave birth to twins were kept on the improved pastures with their lambs, then moved to the semi-improved pastures when the grass in those areas grew up to 4 cm in height. These ewes were eventually moved to the hill ground with their lambs, whereas twin-bearing ewes

managed under the Park Grazing system were retained on the improved pastures or moved to the semi-improved pastures, depending on the availability of the grass in those areas. All the triplet-bearing ewes were kept on the in-bye fields with their lambs, until weaning.

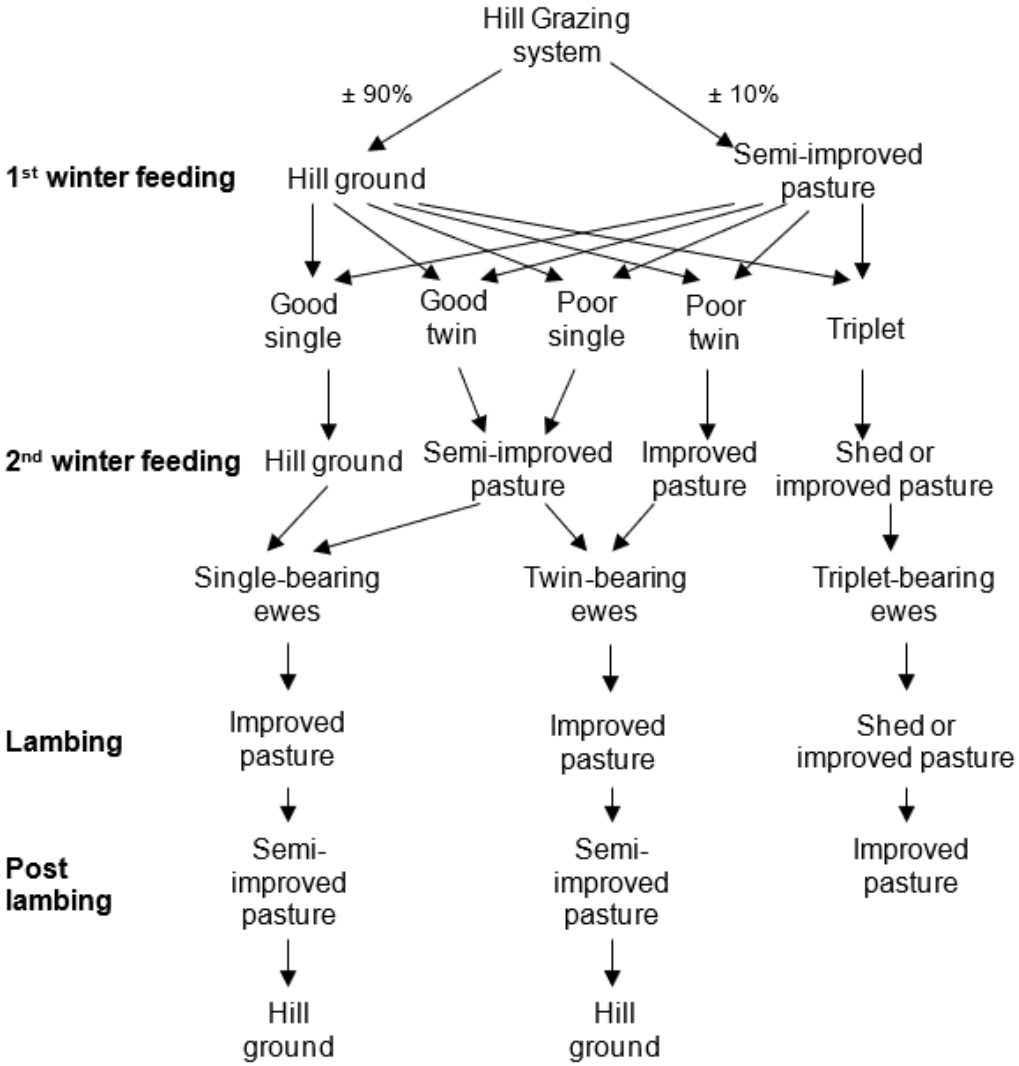


Figure 3-1. Flowchart of winter feeding, type of land used for lambing, and farming ewes and lambs post lambing for the Hill Grazing system, according to their litter size. Top 90% of ewes (determined by weight change of individual ewe, between the January weighing for decision making of winter feeding level and the pre-mating handling event) in this management system were farmed in hill ground during mid-pregnancy, while the remaining 10% of ewes were kept on semi-improved pasture during the same period.

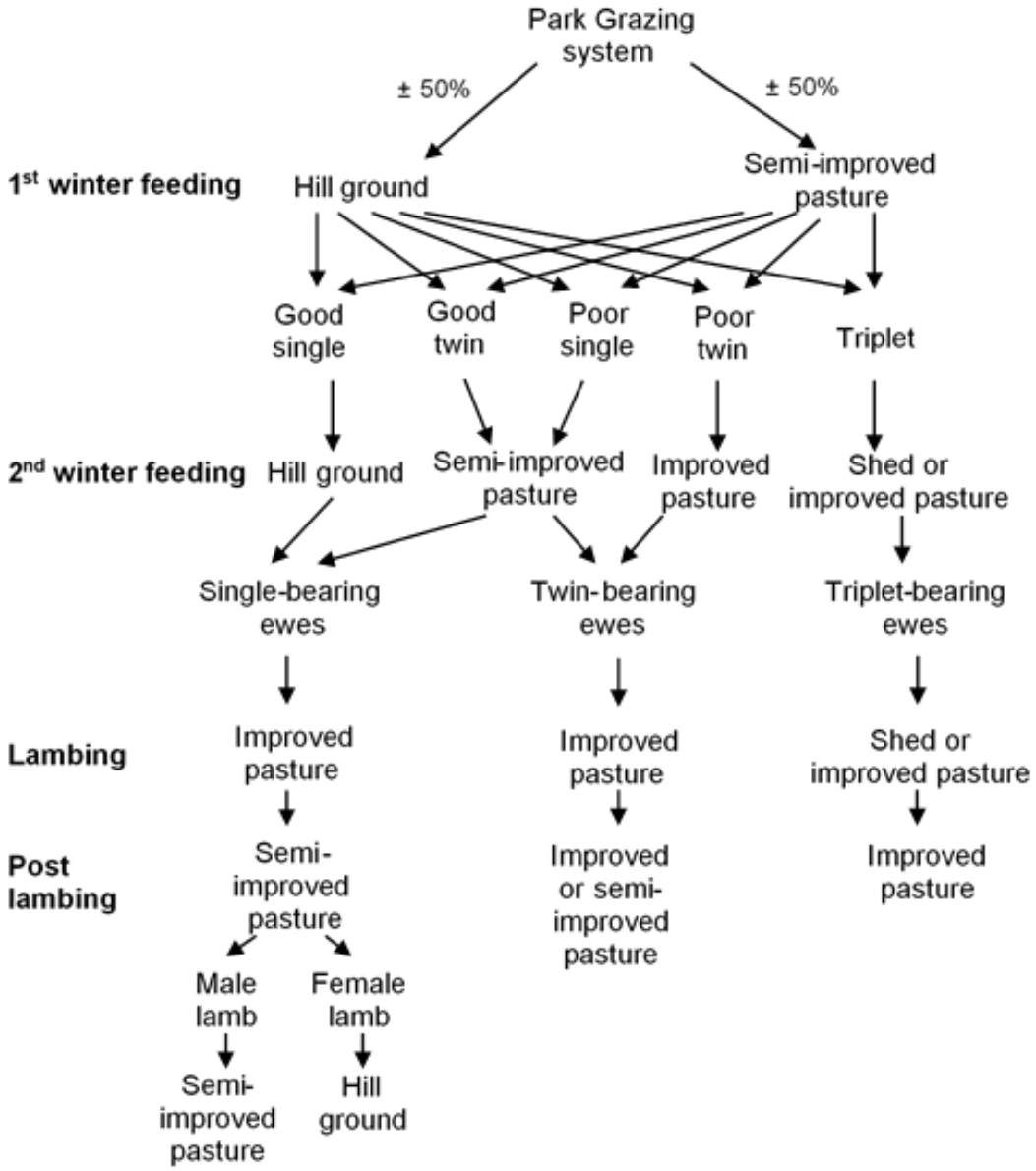


Figure 3-2. Flowchart of winter feeding, type of land used for lambing, and farming ewes and lambs post lambing for the Park Grazing system, according to their litter size. Top 50% of ewes (determined by weight change of individual ewe, between the January weighing for decision making of winter feeding level and the pre-mating handling event) in this management system were farmed in hill ground during mid-pregnancy, while the remaining 50% of ewes were kept on semi-improved pasture during the same period.

All the experiments were conducted in accordance with UK legislation under the Animals (Scientific Procedures) Act 1986. The experimental protocols involving animals were approved by the SRUC Animal Welfare and Ethical Review Body.

The data for investigating ewe and lamb performance were collected in the same manner as described in Section 2.3.3.

3.3.1 Pre-lambing metabolic profile (March 2016 and March 2017)

The collection of plasma samples, and examination of the concentrations of BOHB, albumin, urea N, copper and magnesium from twin-bearing ewes were as described in Section 2.3.4. During late pregnancy, blood samples were taken on the 21st March in 2016 (60 samples; Table 3-1) and on the same date in 2017 (62 samples; Table 3-1). There were not enough suitable ewes from the corrective feeding group under the Park Grazing system, so the numbers were made up with extra ewes selected from the standard feeding group under that management system.

Table 3-1. Distribution, in terms of management system and second winter feeding level, of twin-bearing ewes selected for pre-lambing metabolic profile in 2016 and 2017.

		Management system			
		Hill Grazing		**Park Grazing**	
Year	**Feeding level**	**Standard**	**Corrective**	**Standard**	**Corrective**
2016	**UBF**	5	4	10	1
	IBF	5	8	5	2
	Lleyn	5	7	5	3
2017	**UBF**	6	5	7	4
	IBF	5	6	5	4
	Lleyn	4	6	8	2

Only data of ewes that gave birth to twins and lambed within 25-46 days after blood sampling were statistically analysed. This narrowed time scale matched the recommended 'ideal blood sampling time' (Dairy Herd Health and Productivity Service, 2014) and enabled appropriate comparisons with reference ranges. The statistical analyses included 52 ewes (19 UBF, 19 IBF and 14 Lleyn) in 2016 and 54 ewes (19 UBF, 17 IBF and 18 Lleyn) in 2017.

3.3.2 Colostrum quality in the 2016 and 2017 lambing seasons

Seventy-eight (22 UBF, 29 IBF and 27 Lleyn) and sixty-eight (17 UBF, 28 IBF and 23 Lleyn) colostrum samples (one per ewe) were collected from twin-bearing ewes

in 2016 (between 19th April and 6th May) and 2017 (between 21st April and 9th May) lambing seasons, respectively.

The quality of each fresh colostrum sample was measured using an optical Brix refractometer, at ambient temperature of 20°C. The scale, shown as Brix percentage, ranged from 0 to 32% on the instrument. Each colostrum sample was diluted with distilled water (10 times dilution) using a 1 ml syringe, in order to provide a reading within the range of the instrument. After placing one or two drops of diluted sample onto the refractometer's prism, the refractometer was held against a light source to obtain the result. After each measurement, the prism was washed with tap water. The instrument was then calibrated with distilled water, and the blue line was adjusted to the zero value on its scale. Each diluted colostrum sample provided two Brix percentage readings. The mean of the two measurements was used for data analysis.

In the 2016 lambing season, the lambing times of twin-bearing ewes were observed and recorded (lambing time and ewe neckband number) in the morning, between 3 and 9 am, when twin-bearing ewes were kept in the shed. In the 2017 lambing season, the lambing times of twin-bearing ewes were recorded using a Hikvision camera (Hikvision Digital Technology Ltd, UK), when ewes were kept in the shed overnight.

Only data of ewes that were sampled within 6 hours after lambing (ranged from 30 minutes to 5 hours 56 minutes) were used in the statistical analysis. The lambing time used was the time when the first lamb was born. The data analysis included 38 twin-bearing ewes in 2016 (14 UBF, 12 IBF and 12 Lleyn) and 28 twin-bearing ewes in 2017 (9 UBF, 10 IBF and 9 Lleyn).

3.3.3 Post mortem examination in the 2016 and 2017 lambing seasons

Fifty-three (12 UBF, 28 IBF and 13 Lleyn) and thirty-nine (9 UBF, 19 IBF and 11 Lleyn) lambs were examined in 2016 and 2017 lambing seasons, respectively, following the procedures described in Section 2.3.6.

3.3.4 Pelvic width

On the 19th and 20th September 2016, 689 ewes (239 UBF, 246 IBF and 204 Lleyn) had their external pelvic widths measured. Among these ewes, 48 ewes (11 UBF, 15 IBF and 22 Lleyn) were randomly selected for a 2nd pelvic width measurement in the afternoon of 20th September 2016, in order to examine the repeatability of the method.

The method used for determination of pelvic width was an adaptation of the method demonstrated by Adam (2014). The external pelvic width was measured using a non-invasive calliper. On ewes that were held so that they stood straight, the calliper was placed over the left and the right tuber coxae, then the 'wings' of the calliper were pushed tightly against the surface of the wool for the measurement (Figure 3-3).

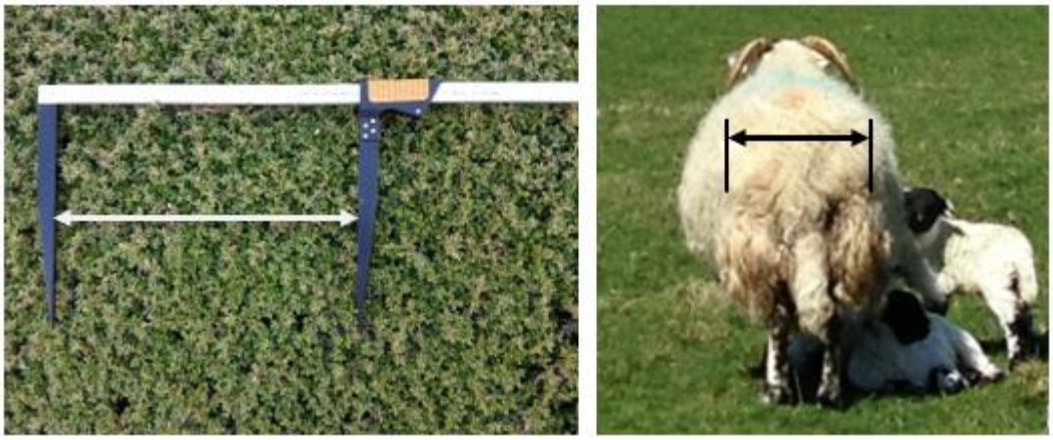


Figure 3-3. Measurement of pelvic width using a calliper.

The data set of ewe pelvic widths was merged with the records of ewe lambing difficulty (assisted (10 UBF, 18 IBF and 17 Lleyn) or non-assisted lambing (164 UBF, 163 IBF and 153 Lleyn)) from the following spring (2017), and relevant fixed effects and covariates were included, to examine the effect of ewe external pelvic width on lambing difficulty.

3.3.5 Data analysis

Data were collated, and ewes that had lamb(s) fostered on or off and those with crossbred lambs were removed from the data file. The one exception was the data file used to analyse barren rate at pregnancy scanning, for which the data file considered all the ewes that were presented at the pregnancy scanning handling event, including those that subsequently gave birth to crossbred lambs or had lambs fostered on or off. The numbers of records in the data files for ewe and lamb performance are summarised in Table 3-2.

Table 3-2. Summary of sample size for the ewe and lamb performance traits (2 years).

Performance trait	UBF	IBF	Lleyn
Number of lambs scanned per ewe mated	352	329	302
Number of lambs born or weaned per ewe mated	353	330	303
Number of lambs weaned per ewe lambed	301	274	256
Barren rate (at scanning)*	391	390	356
Litter birth weight (excluding ewes that did not lamb)	301	274	256
Average lamb birth weight (excluding ewes that did not lamb)	301	274	256
Weaned litter weight (excluding ewes that did not lamb)	301	274	256
Average lamb weaning weight (ewes that weaned lambs)	276	246	237
Lamb growth rate between birth and marking	380	376	374
Lamb growth rate between marking and weaning	371	367	365

*The sample size for barren rate at scanning included all the ewes that were presented at pregnancy scanning.

All the statistical analyses were conducted using GenStat 16 statistical package (VSN International Ltd, UK), except the analysis of the repeated measurements of ewe external pelvic widths, that was conducted using Minitab 17 software. Most of the analyses of ewe and lamb performance among the three genetic lines were investigated using Linear Mixed Model (LMM) via: *1)*, comparing differences in litter size at scanning (based on scanning results, barren ewes included as zero), lambing (ewes that did not lamb included as zero) and weaning (ewes that did not wean lambs included as zero); *2)*, examining differences in litter weight at birth and weaning (excluding ewes that did not lamb) and average lamb weight at birth (excluding ewes that did not lamb) and at weaning (excluding ewes that did not wean lambs); *3)*, assessing lamb growth rate (calculated for average daily gain; g/day) from birth to marking and from marking to weaning. The barren rate at pregnancy scanning (based on scanning results: barren ewe = 0 or non-barren ewe = 1) was analysed using a Generalized Linear Mixed Model (GLMM) with a binomial distribution and a logit link function. The random effects applied in these statistical

analysis models were either ‘year (in this chapter, it was with two levels: 2016 or 2017)’ or ‘sire EID + year’, as explained in Table 3-3.

The fixed effects and relevant covariates applied for investigation of ewe performance were genetic line (UBF, IBF or Lleyn), ewe pre-mating weight (to 0.1 kg), ewe pre-mating BCS (to 0.25 score), ewe age (six levels: from 2 to 7+ years old; 7+ years old included 7-9 years old ewes), first winter feeding level (two levels: standard or corrective), second winter feeding level (two levels: standard or corrective), management system (two levels: Hill Grazing or Park Grazing), birth litter sex (9 levels, which represented the sex of all the lambs within the litter at birth; for example, if a ewe gave birth to triplets comprising two male lambs and one female lamb, the birth litter sex would be MaleMaleFemale), lamb age at weaning (to 1 day) and weaned litter sex (9 levels, which represented the sex of all the lambs within the litter at weaning; for example, if a ewe weaned twins comprising one male lamb and one female lamb, her weaned litter sex would be MaleFemale).

The fixed effects and relevant covariates applied for investigation of lamb performance were genetic line (as above), lamb birth weight (to 0.1 kg), lamb sex (two levels: male or female), litter size at birth (three levels: singleton, twin or triplet), lamb age at marking (to 1 day), number of days between marking and weaning (to 1 day), litter size at weaning (three levels: singleton, twin or triplet), dam age (six levels: from 2 to 7+ years old) and management system (as above). The fixed and random effects applied for each response variate are summarised in Table 3-3.

The statistical analyses of pre-lambing metabolic status were carried out using LMM. Firstly, Generalized Linear Model (GLM) was performed to determine the best fixed effect model, using multiple independent variables. Genetic line (as above), ewe age (three levels: from 3 to 5 years old), ewe weight (as above) and BCS (as above) at scanning, first winter feeding level (as above), second winter feeding level (as above), number of days between sampling and lambing (to 1 day), litter birth weight (to 0.1 kg) and management system (as above) were applied in the Maximal Model in the GLM analysis, for each assayed metabolite. The fixed models, suggested by stepwise GLM regression, for each metabolite and random effect applied in the LMM analyses are summarised in Table 3-3.

Colostrum quality of twin-bearing ewes, measured as Brix percentage, was statistically analysed using LMM. Firstly, GLM was used to determine the best fixed effects model, using multiple independent variables. Genetic line (as above), ewe age (five levels: 2 years old to 6+ years old), time between lambing and sampling (to 1 minute), ewe weight (as above) and BCS (as above) at scanning, first winter feeding level (as above), second winter feeding level (two levels: standard or corrective for twin-bearing ewe), litter birth weight (to 0.1 kg), management system (as above) and year (two levels: 2016 or 2017) were considered in the Maximal Model in the GLM analysis. The variables suggested by stepwise GLM regression for the fixed model in LMM analysis and random effect applied are summarised in Table 3-3.

The effect of genetic line (pure-bred lambs only) on the incidence of lambs dying of dystocia, as diagnosed by post-mortem, was determined using GLMM, with a binomial distribution and a logit link function. GLM analysis tested the effect of lamb genetic line (as above), dam age (six levels: from 2 to 7 years old), lamb birth weight (as above), litter size at birth (as above), lamb sex (as above), lambing date (converted to the number of days after official start of lambing date in that year), ewe pre-mating weight (as above), ewe pre-mating BCS (as above), management system (as above) and year (as above) on this response variate. The fixed model and random model used in the GLMM were based on the suggestion of the stepwise regression (Table 3-3). The birth weights of lambs examined post-mortem were statistically analysed using LMM (Table 3-3). The factors considered as fixed effects were genetic line (as above), dam age (as above), litter size at birth (as above), lamb sex (as above) and cause of lamb death (four levels: dystocia, starvation/hypothermia, other known causes, or inconclusive diagnosis), with ‘year’ as a random effect.

The measurements of ewe external pelvic width (based on the first measurement for all ewes) were statistically analysed using GLM. The maximal model examined the effects of genetic line (as above), ewe age (six levels: from 1.5 to 6.5+ years old when measurements were taken; 6.5+ years old included 6.5 to 9.5 years old ewes), ewe weight at weaning (to 0.1 kg), ewe BCS at weaning (to 0.25 score), ewe weight at the handling event in September when the external pelvic width was measured (to 0.1 kg) and management system (as above). The final model was determined using

a stepwise regression (Table 3-3). The repeated measures of external pelvic width, measured twice on the same ewes, were compared using Pearson correlation tests. The differences between the two sets of measurements, assessed as absolute values, were analysed using one-way ANOVA to determine the effect of genetic line. In order to assess the effect of ewe external pelvic width on lambing difficulty (non-assisted lambing = 0 or assisted lambing = 1), GLM was used to investigate the effects of multiple independent variables and covariates. The variables and covariates considered in the maximal model were genetic line (as above), ewe age (six levels: from 1.5 to 6.5+ years old), ewe external pelvic width (to 0.1 cm), litter birth sex (as above), litter birth weight (to 0.1 kg), ewe weight and ewe BCS at scanning (as above) and management system (as above). The fixed model used in the GLMM (with a binomial distribution and a logit link function) was determined based on the suggestion of the stepwise regression (Table 3-3).

For all the response variates examined in this chapter, if genetic line and management system were both considered in the statistical analysis model, the interaction effect of these two factors was then adjusted in the statistical analysis to determine the differences between the two management systems for the three genetic lines (Table 3-3). The only exception was the statistical analysis of magnesium plasma status, for which, the LMM model that includes interaction effect of genetic line and management system would not converge.

Statistical significance was defined as $P < 0.05$. When model terms were significant, pairwise Student's t-tests were performed to test for significant differences between different levels of each factor.

Table 3-3. Summary of statistical analyses applied, with corresponding type of the analysis and final models with fixed effects and random effects applied for each response variate.

Response variate category	Response variate	Model	Fixed model for LMM or GLMM / Model to be fitted for GLM	Random model
Ewe performance	Number of lambs scanned per ewe mated	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + management system	Year
		LMM	Genetic line + management system + geneitc line x management system + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level	Year
	Number of lambs born per ewe mated	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + management system	Year
		LMM	Genetic line + management system + genetic line x management system + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level	Year
	Number of lambs weaned per ewe mated	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + management system	Year
		LMM	Genetic line + management system + genetic line x management system + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level	Year
	Number of lambs weaned per ewe lambed	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + management system	Year
		LMM	Genetic line + management system + genetic line x management system + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level	Year
	Barren rate (at scanning)	GLMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + management system	Year
		GLMM	Genetic line + management system + genetic line x management system + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level	Year
	Litter birth weight (excluding ewes that did not lamb)	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + second winter feeding level + management system	Year
		LMM	Genetic line + management system + genetic line x management	Year

Response variate category	Response variate	Model	Fixed model for LMM or GLMM / Model to be fitted for GLM	Random model
			system + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + second winter feeding level	
	Average lamb birth weight (excluding ewes that did not lamb)	LMM	Genetic line + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + second winter feeding level + birth litter sex + management system	Year
		LMM	Genetic line + management system + genetic line x management system + ewe pre-mating weight + ewe pre-mating BCS + ewe age + first winter feeding level + second winter feeding level + birth litter sex	Year
	Weaned litter weight (excluding ewes that did not lamb)	LMM	Genetic line + ewe pre-mating weight + ewe age + first winter feeding level + second winter feeding level + lamb age at weaning + management system	Year
		LMM	Genetic line + management system + genetic line x management system + ewe pre-mating weight + ewe age + first winter feeding level + second winter feeding level + lamb age at weaning	Year
	Average lamb weaning weight (ewes that had lambs weaned)	LMM	Genetic line + ewe pre-mating weight + ewe age + first winter feeding level + second winter feeding level + lamb age at weaning + weaned litter sex + management system	Year
		LMM	Genetic line + management system + genetic line x management system + ewe pre-mating weight + ewe age + first winter feeding level + second winter feeding level + lamb age at weaning + weaned litter sex	Year
Lamb performance	Lamb growth rate between birth and marking	LMM	Genetic line + lamb birth weight + lamb sex + litter size at birth + lamb age at marking + dam age + management system	Sire EID + year
		LMM	Genetic line + management system + genetic line x management system + lamb birth weight + lamb sex + litter size at birth + lamb age at marking + dam age	Sire EID + year
	Lamb growth rate between marking and weaning	LMM	Genetic line + lamb birth weight + lamb sex + litter size at weaning + number of days between marking and weaning + lamb age at marking + dam age + management system	Sire EID + year
		LMM	Genetic line + management system + genetic line x management system + lamb birth weight + lamb sex + litter size at weaning + number of days between marking and weaning + lamb age at	Sire EID + year

Response variate category	Response variate	Model	Fixed model for LMM or GLMM / Model to be fitted for GLM	Random model
			marking + dam age	
Pre-lambing metabolic profile	BOHB concentration	LMM	Genetic line + ewe weight at scanning + ewe BCS at scanning + first winter feeding level +number of days between sampling and lambing + litter birth weight + management system	Year
		LMM	Genetic line + management system + genetic line x management system + ewe weight at scanning + ewe BCS at scanning + first winter feeding level +number of days between sampling and lambing + litter birth weight	Year
	Albumin concentation	LMM	Genetic line + ewe age + ewe weight at scanning + ewe BCS at scanning + second winter feeding level + litter birth weight + management system	Year
		LMM	Genetic line + management system + genetic line x management system + ewe age + ewe weight at scanning + ewe BCS at scanning + second winter feeding level + litter birth weight	Year
	Urea N concentration	LMM	Genetic line + ewe weight at scanning + number of days between sampling and lambing	Year
	Copper concentration	LMM	Genetic line + ewe age + first winter feeding level + litter birth weight	Year
	Magnesium concentration	LMM	Genetic line + ewe BCS at scanning + management system	Year
Colostrum quality	Brix percentage	LMM	Genetic line + ewe age + ewe weight at scanning + ewe BCS at scanning + first winter feeding level + litter birth weight + management system	Year
		LMM	Genetic line + management system + genetic line x management system + ewe age + ewe weight at scanning + ewe BCS at scanning + first winter feeding level + litter birth weight	Year
Post mortem examination	Died of dystocia as a consequence	GLMM	Genetic line + dam age + lamb birth weight + litter size at birth + lamb sex + lambing date + ewe pre-mating weight + ewe pre-mating BCS	Year
	Lamb birth weight	LMM	Genetic line + dam age + litter size at birth + lamb sex + cause of lamb death	Year
Ewe pelvic	Ewe external pelvic	GLM	Genetic line + ewe age + ewe weight at the measurement of	-

Response variate category	Response variate	Model	Fixed model for LMM or GLMM / Model to be fitted for GLM	Random model
dimension	width		external pelvic width + management system	
		GLM	Genetic line + management system + genetic line x management system + ewe age + ewe weight at the measurement of external pelvic width	-
	Lambing difficulty (assisted or non-assisted at lambing	GLMM	Genetic line + ewe age + ewe external pelvic width + litter birth sex + litter birth weight + ewe BCS at scanning + management system	Sire EID
		GLMM	Genetic line + management system + genetic line x management system + ewe age + ewe external pelvic width + litter birth sex + litter birth weight + ewe BCS at scanning	Sire EID

3.4 Results

3.4.1 Ewe reproductive performance (Nov 2015 to Oct 2017)

The unadjusted results in terms of ewe and lamb performance for UBF, IBF and Lleyn are shown in Table 3-4. The percentages of ewes in the different litter size categories at birth and weaning are shown in Table 3-5.

Table 3-4. Ewe and lamb performance data (mean ± SD) recorded for the three sheep genetic lines across two consecutive production years.

Animal performance	Production year	UBF	IBF	Lleyn
Ewe mature weight (kg)*	2016-2017	50.44	52.64	49.9
Replacement rate (%)	2015-2016	23	22	20
	2016-2017	35	36	36
Ewe pre-mating weight (kg ± SD)	2015-2016	53.7 ± 5.6	57.2 ± 5.4	53.6 ± 6.9
	2016-2017	53.0 ± 5.6	56.5 ± 5.9	52.6 ± 6.5
Ewe pre-mating BCS (± SD)	2015-2016	3.0 ± 0.3	2.9 ± 0.3	2.8 ± 0.3
	2016-2017	2.9 ± 0.3	3.1 ± 0.4	2.7 ± 0.2
Ewe scanning weight (kg ± SD)	2015-2016	46.8 ± 5.5	50.1 ± 6.0	46.9 ± 7.0
	2016-2017	49.4 ± 5.7	53.2 ± 6.1	50.1 ± 7.0
Ewe scanning BCS (± SD)	2015-2016	2.8 ± 0.3	2.7 ± 0.3	2.6 ± 0.3
	2016-2017	2.8 ± 0.2	2.8 ± 0.2	2.7 ± 0.3
Ewe pre-lambing weight (kg ± SD)	2015-2016	50.7 ± 7.5	56.5 ± 7.6	53.4 ± 9.9
	2016-2017	51.6 ± 7.1	56.8 ± 7.9	53.1 ± 9.1
Barren rate at pregnancy scanning (%)	2015-2016	11	10	12
	2016-2017	8	8	5
Ewe mortality (%)	2015-2016	5.1	3.0	3.2
	2016-2017	2.5	2.0	2.2
Litter birth weight per ewe mated (kg ± SD)	2015-2016	4.3 ± 2.3	4.9 ± 2.7	5.0 ± 3.3
	2016-2017	4.5 ± 2.4	5.0 ± 2.8	5.6 ± 2.7
Litter birth weight per ewe lambed (kg ± SD)	2015-2016	5.1 ± 1.5	5.9 ± 1.7	6.3 ± 2.3
	2016-2017	5.2 ± 1.7	6.0 ± 1.8	6.2 ± 2.0
Average lamb birth weight per ewe lambed (kg ± SD)	2015-2016	3.9 ± 0.7	4.0 ± 0.7	4.0 ± 0.8
	2016-2017	3.7 ± 0.6	3.9 ± 0.7	4.2 ± 0.8
Weaned litter weight per ewe mated (kg ± SD)	2015-2016	24.4 ± 16.8	29.1 ± 21.6	28.8 ± 24.6
	2016-2017	28.3 ± 19.0	30.6 ± 22.2	34.1 ± 21.4
Weaned litter weight per ewe that weaned lamb(s) (kg ± SD)	2015-2016	31.4 ± 11.9	39.2 ± 15.2	42.2 ± 17.8
	2016-2017	36.1 ± 13.3	40.9 ± 15.4	39.4 ± 17.9

* Ewe mature weight was estimated by standardizing pre-mating weights to a BCS of 2.75 and ewe age to 3 years old (Carson et al., 2001a).

Table 3-5. Distribution of ewes by litter size at birth and weaning for UBF, IBF and Lleyn ewes based on two oestrous cycles in the presence of rams of their own genetic line.

		Litter size							
		Birth				Weaning			
Year	**Genotype**	**0***	**1**	**2**	**3**	**0***	**1**	**2**	**3**
	UBF	16%	54%	31%	0%	22%	56%	22%	0%
2016	**IBF**	16%	40%	43%	1%	26%	43%	30%	1%
	Lleyn	22%	35%	40%	4%	32%	30%	36%	2%
	UBF	13%	49%	37%	1%	21%	47%	31%	1%
2017	**IBF**	18%	36%	43%	3%	25%	39%	34%	2%
	Lleyn	10%	47%	39%	4%	14%	50%	36%	3%

*percentages in these columns were based on just two oestrous cycles. Some of those ewes became pregnant in the subsequent oestrous cycle. Lower barrenness data in Section 3.4.1.1 reflect this.

3.4.1.1 Litter size

The barren rate at pregnancy scanning did not differ among the three genetic lines (P=0.910; predicted means: UBF = 9%, IBF = 9%, Lleyn = 8%), after adjusting for ewe pre-mating weight (P=0.566), ewe pre-mating BCS (P=0.677), ewe age (P=0.426), first winter feeding level (P<0.001; standard feeding = 5%, corrective feeding = 15%) and management system (P=0.037; Hill Grazing = 11%, Park Grazing = 6%). When the interaction effect of genetic line and management system was fitted into the statistical analysis model, it did not affect the barren rate at pregnancy scanning (P=0.363), after adjusting for ewe pre-mating weight (P=0.483), ewe pre-mating BCS (P=0.650), ewe age (P=0.487) and first winter feeding level (P<0.001; predicted means: standard feeding = 5%, corrective feeding = 15%).

Genetic line (UBF, IBF and Lleyn) was not a significant determinant of litter size at pregnancy scanning (number of foetuses per ewe mated with rams of their own genetic line; P=0.062; Figure 3-4), when the model was adjusted for ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P=0.512), ewe age (P=0.033), first winter feeding level (P<0.001) and management system (P=0.150). Ewe pre-mating weight was positively associated with litter size at pregnancy scanning. Ewes fed with the standard feeding level in the first winter feeding phase had greater litter size than their counterparts fed with the corrective feeding level in the same phase (P<0.001). At ultrasound pregnancy scanning, the litter size of 6 years old ewes was significantly greater than those of 3 and 5 years old ewes (P<0.05 and P<0.01, respectively), while the litter size of 4 years old ewes was higher than that of 5 years old ewes (P<0.05). When the interaction of genetic line and management system

was considered in the statistical analysis model, it did not have an effect on the litter size at pregnancy scanning (P=0.518), after adjusting for ewe pre-mating weight (P<0.001; positive association), ewe pre-mating BCS (P=0.510), ewe age (P=0.039; same effects as demonstrated above) and first winter feeding level (P<0.001; predicted means: standard feeding = 1.42, corrective feeding = 1.18).

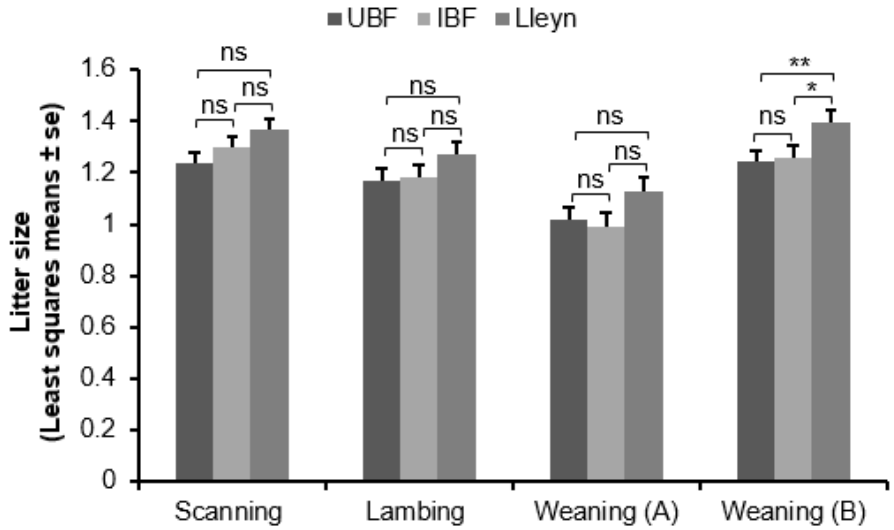


*Figure 3-4. The litter sizes at scanning, lambing and weaning (least squares means ± se) for the three genetic lines. Bars for Scanning, Lambing and Weaning (A) represent the litter size per ewe mated, while bars for Weaning (B) represent the litter size per ewe lambed. ns indicates not significant (P>0.05); * indicates significance at P<0.05; ** indicates significance at P<0.01; *** indicates significance at P<0.001. Same symbols for denoting the level of significance are used in all Figures in this chapter.*

There was no significant difference in the number of lambs born per ewe mated among the three genetic lines (P=0.190; Figure 3-4), after adjusting for ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P=0.197), ewe age (P=0.043), first winter feeding level (P<0.001) and management system (P=0.035; predicted means: Hill Grazing = 1.15, Park Grazing = 1.26). Ewe pre-mating weight was positively associated with the number of lambs born in the following spring. Ewes that received the standard feeding level during the first winter feeding period had more lambs born than those that received the corrective feeding level in the same period (P<0.001). At lambing, the litter size of 5 years old ewes was significantly smaller than those of 2 years, 4 years and 6 years old ewes (P<0.05, P<0.01 and P<0.01, respectively). When the interaction effect of genetic line and management system

was fitted into the LMM analysis model, it was not a significant determinant of the number of lambs born per ewe mated (P=0.518), after adjusting for ewe pre-mating weight (P<0.001; positive association), ewe pre-mating BCS (P=0.194), ewe age (P=0.047; same effects as aforementioned) and first winter feeding level (P<0.001; predicted means: standard feeding = 1.38, corrective feeding = 1.04).

At weaning, there was no significant difference in the number of lambs weaned per ewe mated among the three genetic lines (P=0.083; Figure 3-4), after adjusting for ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P=0.054), ewe age (P=0.383), first winter feeding level (P<0.001) and management system (P=0.052). The number of lambs weaned per ewe mated was positively associated with ewe pre-mating weight. Ewes fed with the standard feeding level in the first winter feeding period weaned more lambs per ewe mated, compared to counterparts fed with the corrective feeding level in the same period (P<0.001). When the interaction of genetic line and management system was considered in the statistical analysis model, it did not have a significant effect on the number of lambs weaned per ewe mated (P=0.502), after adjusting for ewe pre-mating weight (P<0.001; positive association), ewe pre-mating BCS (P=0.049; negative association; BCS ranged from 2.25 to 4.0), ewe age (P=0.398) and first winter feeding level (P<0.001; predicted means: standard feeding = 1.19, corrective feeding = 0.91).

After removing the ewes that did not lamb from the data set, the number of lambs weaned per Lleyn ewe lambed was significantly greater than in the cases of UBF and IBF ewes (P<0.01 and P<0.05, respectively; Figure 3-4). Ewe pre-mating weight was positively associated with the number of lambs weaned per ewe lambed (P<0.001). Ewe pre-mating BCS (P=0.303), ewe age (P=0.542), first winter feeding level (P=0.916) and management system (P=0.865) did not have significant effects on the number of lambs weaned per ewe lambed. When the interaction effect of genetic line and management system was fitted into the statistical analysis model, it was not a significant determinant of the number of lambs weaned per ewe that lambed (P=0.502), after adjusting for ewe pre-mating weight (P<0.001; positive association), ewe pre-mating BCS (P=0.295), ewe age (P=0.538) and first winter feeding level (P=0.928).

3.4.1.2 Birth weight

After excluding the ewes that did not lamb from the data set, Lleyn ewes had significantly heavier litter birth weight per ewe lambed than UBF and IBF ewes (P<0.001 and P<0.001, respectively; Figure 3-5), and IBF ewes had greater litter birth weight per ewe lambed than UBF ewes (P<0.01), after adjusting for ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P=0.442), ewe age (P=0.013), first winter feeding level (P=0.809), second winter feeding level (P=0.605) and management system (P=0.186). Ewe pre-mating weight was positively associated with litter birth weight per ewe lambed. The litters born from 4 years old ewes were heavier than those born from gimmers and 5 years old ewes (P<0.05 and P<0.05, respectively). When the interaction effect of genetic line and management system was considered into the LMM model, it did not significantly affect litter birth weight per ewe that lambed (P=0.502), after adjusting for ewe pre-mating weight (P<0.001; positive association), ewe pre-mating BCS (P=0.462), ewe age (P=0.01; same effects as aforementioned), first winter feeding level (P=0.815) and second winter feeding level (P=0.656).

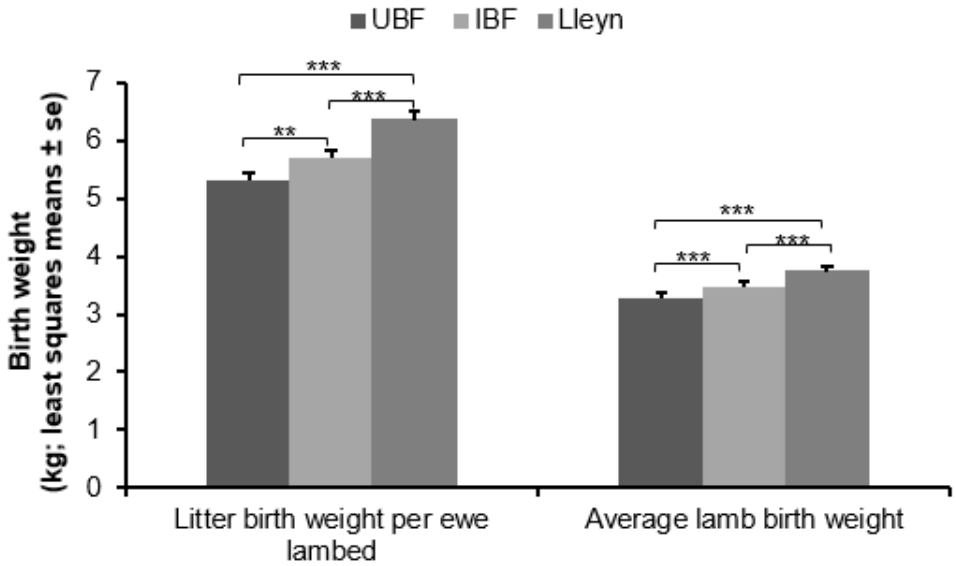


Figure 3-5. The litter birth weight and the average lamb birth weight (kg; least squares mean ± se) for the three genetic lines, when based on the ewes that gave birth to lamb(s).

The LMM analysis of average lamb birth weight (based on ewes that lambed) showed that Lleyn ewes had heavier average lamb birth weight than UBF and IBF

ewes (P<0.001 and P<0.001, respectively; Figure 3-5), IBF ewes had greater average lamb birth weight than UBF ewes (P<0.001), when the model was adjusted for ewe pre-mating weight (P<0.001), ewe pre-mating BCS (P=0.909), ewe age (P<0.001), first winter feeding level (P=0.374), second winter feeding level (P=0.399), birth litter sex (P<0.001) and management system (P=0.018; predicted means: Hill Grazing = 3.44 kg, Park Grazing = 3.55 kg). Ewe pre-mating weight was positively associated with average lamb birth weight. The average lamb birth weight among gimmers was significantly lighter than among mature ewes (P<0.001, P<0.001, P<0.001, P<0.001 and P<0.01 for 2 *vs*. 3, 2 *vs*. 4, 2 *vs.* 5, 2 *vs.* 6 and 2 *vs*. 7+ years old ewes, respectively). The average lamb birth weight of male singletons was heavier than that of female counterparts (P<0.01; predicted means: male = 4.43 kg, female = 4.28 kg), whereas no effects of birth litter sex on the average lamb birth weights were found among twin or triplet litters. When the interaction effect of genetic line and management system was fitted into the statistical analysis model, it was a significant determinant of the average lamb birth weight (P=0.044), after adjusting for ewe pre-mating weight (P<0.001; positive association), ewe pre-mating BCS (P=0.966), ewe age (P<0.001; same effects as described above), first winter feeding level (P=0.349), second winter feeding level (P=0.309) and birth litter sex (P<0.001; same effect at aforementioned). For ewes being managed under the Hill Grazing system, the average lamb birth weights of Lleyn and IBF ewes were heavier than that of UBF ewes (P<0.001 and P<0.001, respectively; Figure 3-6), while this parameter did not differ significantly between IBF and Lleyn ewes (P>0.05). For ewes being managed under the Park Grazing system, Lleyn ewes had the heaviest average lamb birth weight, followed by IBF and UBF ewes (Figure 3-6). Within each genetic line, the average lamb birth weight of Lleyn ewes managed under the Park Grazing system was heavier than that of their counterparts managed under the Hill Grazing system (P<0.01), while there was no such difference in average lamb birth weight for UBF and IBF ewes (P>0.05).

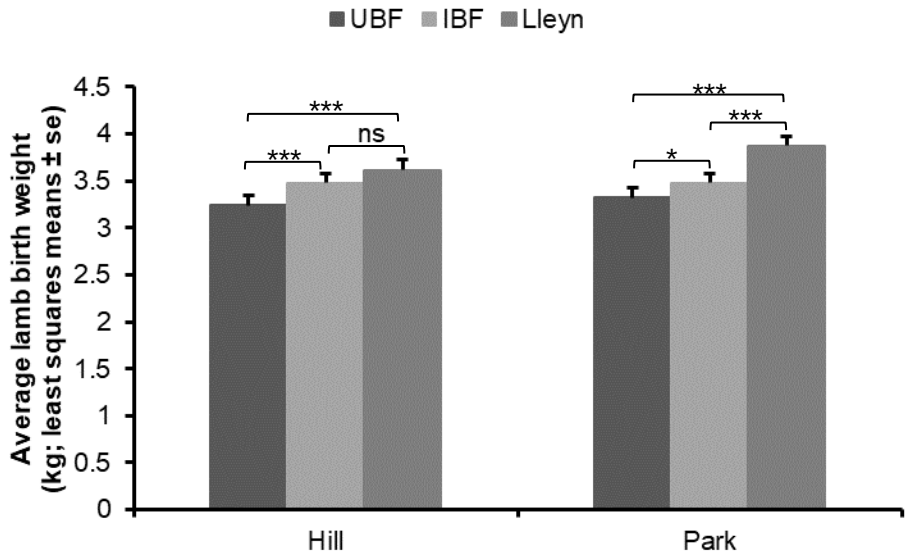


Figure 3-6. The average lamb birth weight (kg; least squares mean ± se) for the three genetic lines, according to their management systems.

3.4.1.3 Weaning weight

The LMM analysis of weaned litter weight per ewe that lambed showed that litters weaned from Lleyn ewes were heavier than those weaned from UBF and IBF ewes (P<0.001 and P<0.001, respectively; Figure 3-7), after adjusting for ewe pre-mating weight (P<0.001), ewe age (P=0.553), first winter feeding level (P=0.879), second winter feeding level (P=0.282), lamb age at weaning (P=0.002) and management system (P=0.004; predicted means: Hill Grazing = 32.15 kg, Park Grazing = 36.05 kg). Ewe pre-mating weight and lamb age at weaning were positively associated with weaned litter weight per ewe that lambed. When the interaction effect of genetic line and management system was considered in the LMM model, it did not significantly affect weaned litter weight per ewe that lambed (P=0.294), after adjusting for ewe pre-mating weight (P<0.001; positive association), ewe age (P=0.527), first winter feeding level (P=0.842), second winter feeding level (P=0.389) and lamb age at weaning (P=0.002; positive association).

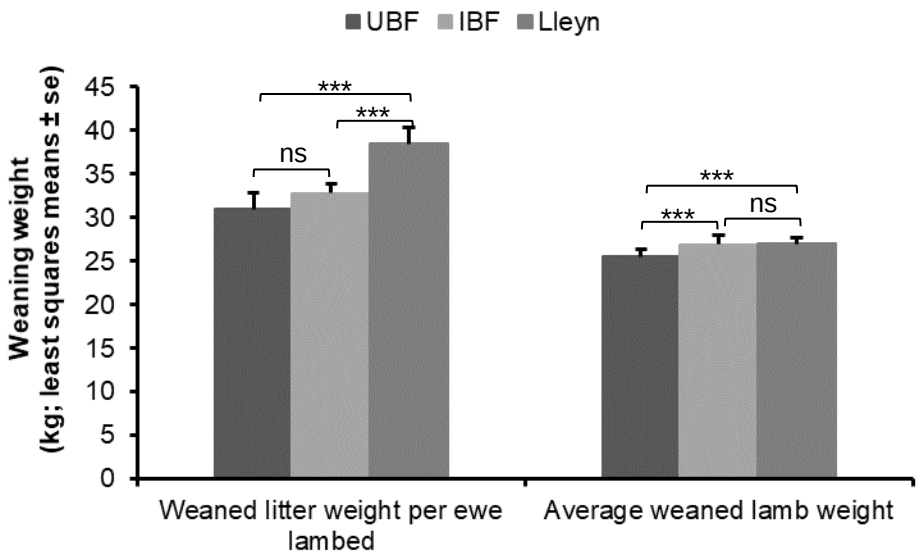


Figure 3-7. The weaned litter weight per ewe that lambed and the average lamb weaning weight per ewe that weaned lambs (kg; lease squares mean ± se) for the three genetic lines.

Among the ewes that had lamb(s) weaned, average lamb weaning weights per ewe that weaned lambs of IBF and Lleyn ewes were similar (P>0.05) and each was greater than that of UBF ewes (P<0.001 and P<0.001, respectively; Figure 3-7), when ewe pre-mating weight (P<0.001), ewe age (P=0.088), first winter feeding level (P=0.357), second winter feeding level (P=0.078), lamb age at weaning (P<0.001), weaned litter sex (P<0.001) and management system (P<0.001; predicted means: Hill Grazing = 25.34 kg, Park Grazing = 27.64 kg) were adjusted for in the LMM model. Ewe pre-mating weight and lamb age at weaning were positively associated with average lamb weaning weight per ewe that weaned lambs. Male lambs weaned as singletons were heavier than their female counterparts (P<0.001; predicted means: male = 27.77 kg, female = 24.79 kg). Average lamb weaning weight per ewe that weaned lambs of male-male twin litters was heavier than that of female-female twin litters (P<0.05; predicted means: male-male = 26.74 kg, female-female = 25.42 kg), but no such difference was found either between male-male and male-female or female-female and male-female twin litters (P>0.05 or P>0.05, respectively; predicted mean: male-female = 25.67 kg). When the interaction effect of genetic line and management system was fitted into the LMM analysis model, it was a significant determinant of average lamb weaning weight per ewe that weaned lambs (P=0.003), after adjusting for ewe pre-mating weight (P<0.001; positive association), ewe age (P=0.067), first winter feeding level (P=0.391), second winter feeding level (P=0.191), lamb age at weaning (P<0.001;

positive association) and weaned litter sex (P<0.001; same effects as demonstrated above). For ewes being managed under the Hill Grazing system, IBF ewes had a heavier average lamb weaning weight per ewe that weaned lambs than UBF ewes (P<0.01), while there was no significant difference in this parameter either between UBF and Lleyn or IBF and Lleyn ewes (P>0.05; Figure 3-8). For ewes being managed under the Park Grazing system, Lleyn and IBF ewes had heavier average lamb weaning weights per ewe that weaned lambs than UBF ewes (P<0.001 and P<0.001, respectively), while this parameter did not differ between IBF and Lleyn ewes (P>0.05; Figure 3-8). Within each genetic line, average lamb weaning weight per ewe that weaned lambs of ewes managed under the Park Grazing system was greater than that of their counterparts managed under the Hill Grazing system (UBF: P<0.01; IBF: P<0.001; Lleyn: P<0.001).

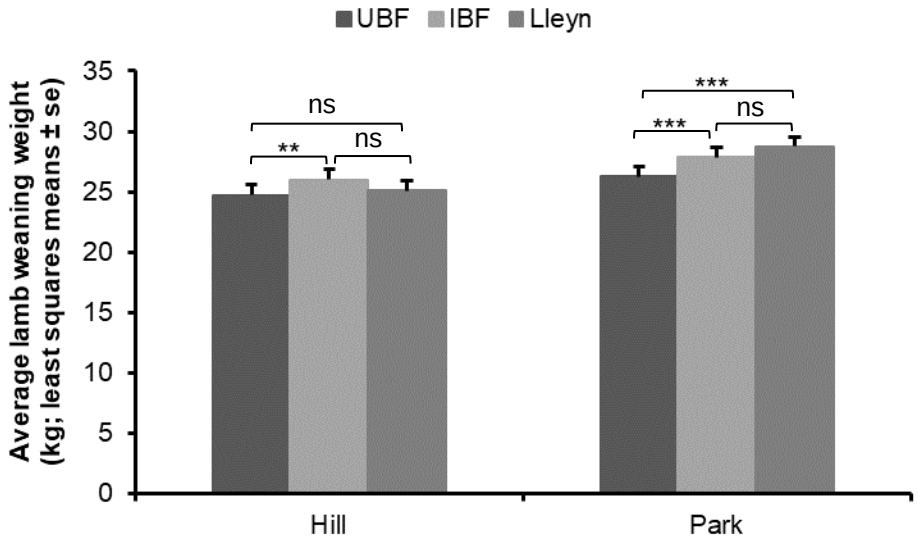


Figure 3-8. The average lamb weaning weight per ewe that weaned lambs (kg; least squares mean ± se) for the three genetic lines, according to their management systems.

3.4.1.4 Lamb growth rate

Between birth and marking, the IBF and Lleyn lambs grew faster than the UBF lambs (P<0.01 and P<0.001, respectively) but lamb growth rate did not differ between IBF and Lleyn lambs (P>0.05; Figure 3-9), when lamb birth weight (P<0.001), lamb sex (P=0.002; predicted means: male = 250.5 g/day, female = 242.5 g/day), litter size at birth (P<0.001), number of days between birth and

marking (P=0.047), dam age (P=0.152) and management system (P=0.535) were accounted for in the model. Lamb birth weight was positively associated with lamb growth rate, while number of days between birth and marking was negatively associated with lamb growth rate. In the 'birth to marking' period, twins and triplets grew faster than singletons (P<0.001 and P<0.01, respectively; Figure 3-10). When the interaction of genetic line and management system was fitted into the corresponding statistical analysis model, it had a significant effect on lamb growth rate in this study period (P=0.001), after adjusting for lamb birth weight (P<0.001; positive association), lamb sex (P=0.002; predicted means: male = 250.2 g/day, female = 242.3 g/day), litter size at birth (P<0.001; same effects as aforementioned), number of days between birth and marking (P=0.071) and ewe age (P=0.175). For lambs managed under the Hill Grazing system, lamb growth rate between birth and marking did not differ among the three genetic lines (P>0.05; Figure 3-11). For lambs managed under the Park Grazing system, IBF and Lleyn lambs grew quicker than UBF lambs (P<0.001 and P<0.001, respectively), while no significant difference was found between IBF and Lleyn lambs (P>0.05; Figure 3-11). Within each genetic line, only UBF lambs managed under the Hill Grazing system grew faster than their counterparts managed under the Park Grazing system (P<0.001), while there was no such difference for IBF and Lleyn lambs (P>0.05).

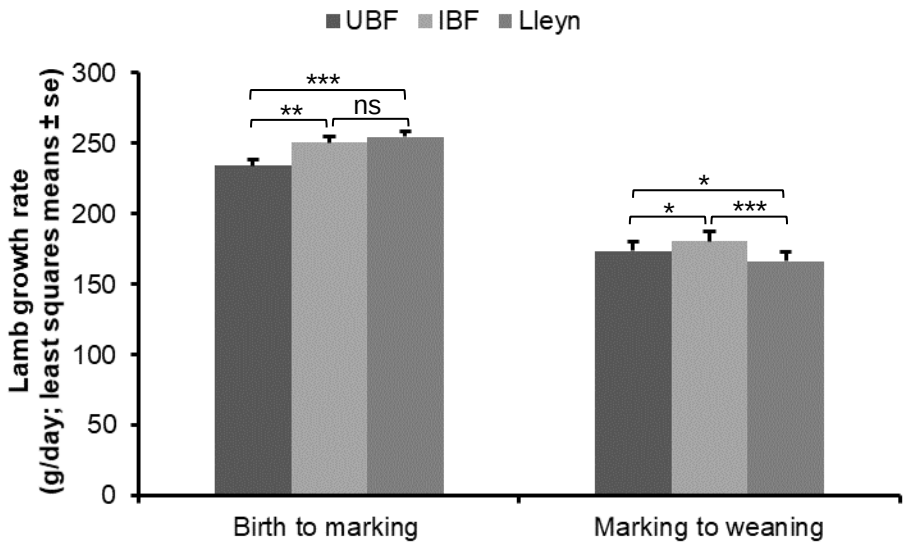


Figure 3-9. Lamb growth rate (g/day; least squares mean ± se) between birth and marking, and between marking and weaning, for the three genetic lines.

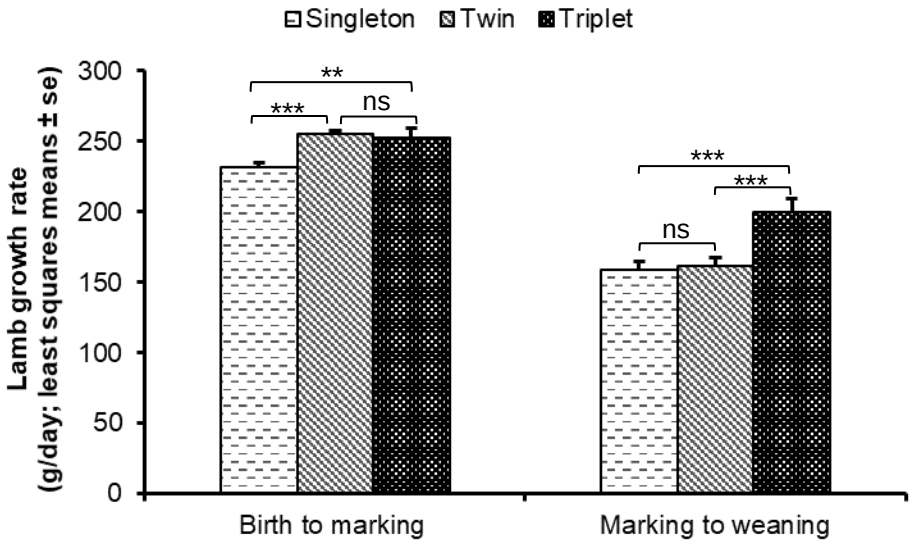


Figure 3-10. Lamb growth rate (g/day; least squares mean ± se) between birth and marking, and between marking and weaning, for singletons, twins and triplets.

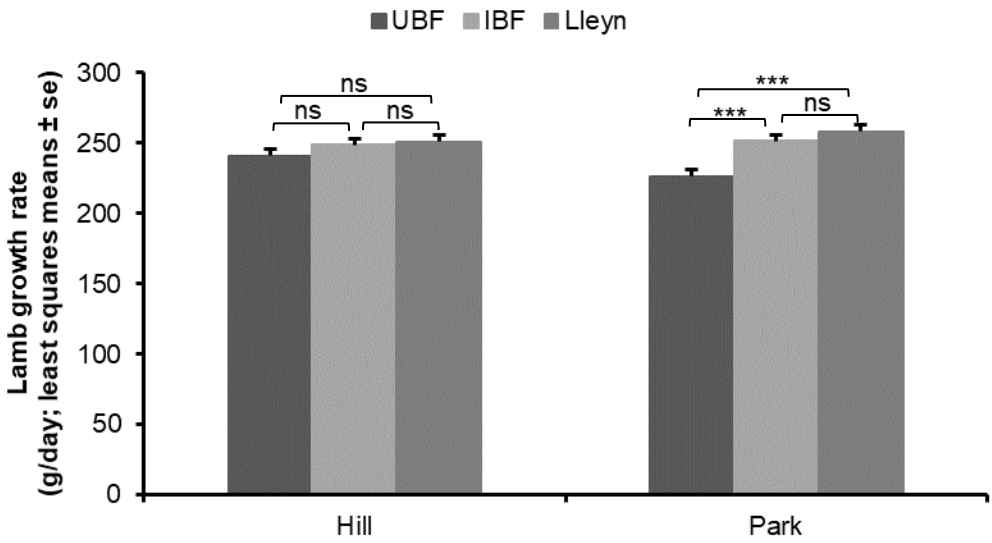


Figure 3-11. Lamb growth rate (g/day; least squares mean ± se) between birth and marking, for the three genetic lines, according to their management systems.

Between marking and weaning, the IBF lambs grew faster than the UBF and Lleyn lambs ($P<0.05$ and $P<0.001$, respectively), and the UBF lambs grew faster than the Lleyn lambs ($P<0.05$; Figure 3-9), the LMM model having been adjusted for lamb birth weight ($P=0.024$), lamb sex ($P<0.001$; predicted means: male = 185.7 g/day, female = 161.2 g/day), litter size at weaning ($P<0.001$), number of days between marking and weaning ($P=0.014$), lamb age at marking ($p=0.059$), dam age

(P=0.427) and management system (P<0.001; predicted means: Hill Grazing = 151.8 g/day, Park Grazing = 195.1 g/day). Lamb birth weight was positively associated with lamb growth rate, while number of days between marking and weaning was negatively associated with lamb growth rate. Between marking and weaning, triplets grew faster than singletons and twins (P<0.001 and P<0.001, respectively). The growth rate did not differ between singletons and twins (P>0.05; Figure 3-10). When the interaction effect of genetic line and management system was considered into the LMM model, it was a significant determinant of lamb growth rate between marking and weaning (P=0.001), after adjusting for lamb birth weight (P=0.054), lamb sex (P=0.002; predicted means: male = 185.4 g/day, female = 160.9 g/day), litter size at weaning (P<0.001; same effects as demonstrated above), number of days between marking and weaning (P=0.007; negative association), lamb age at marking (P=0.077) and dam age (P=0.331). For lambs managed under the Hill Grazing management system, growth rates between marking and weaning of UBF and IBF lambs were higher than that of Lleyn lambs (P<0.001 and P<0.001, respectively), while this parameter did not differ between UBF and IBF lambs (P>0.05; Figure 3-12). For lambs managed under the Park Grazing system, growth rate did not differ among the three genetic lines (P>0.05, Figure 3-12). Within each genetic line, lambs managed under the Park Grazing system grew quicker than their counterparts managed under the Hill Grazing system (P<0.001, P<0.001 and P<0.001 for UBF, IBF and Lleyn lambs, respectively).

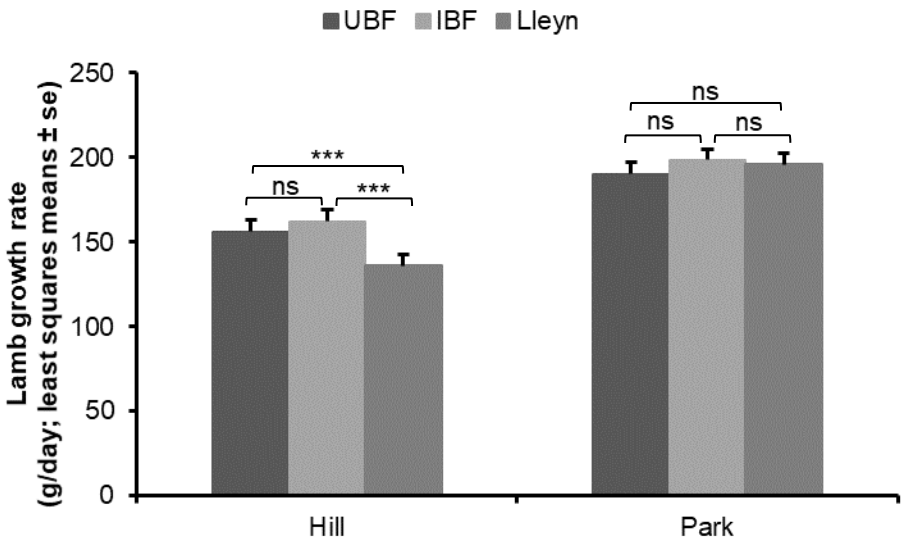


Figure 3-12. Lamb growth rate (g/day; least squares mean ± se) between marking and weaning, for the three genetic lines, according to their management systems.

3.4.2 Pre-lambing metabolic profile (March 2016 and March 2017)

The predicted means of BOHB, albumin, urea N, copper and magnesium plasma concentrations for UBF, IBF and Lleyn twin-bearing ewes were all within the recommended ovine reference ranges (Table 3-6).

UBF and IBF twin-bearing ewes had higher BOHB concentrations than their Lleyn counterparts ($P<0.05$ and $P<0.01$, respectively) but there was no difference in BOHB plasma concentration between UBF and IBF twin-bearing ewes ($P>0.05$), after adjusting for ewe weight ($P=0.737$) and ewe BCS ($P=0.068$) at scanning, first winter feeding level ($P=0.306$), number of days between sampling and lambing ($P=0.073$), litter birth weight ($P=0.018$) and management system ($P=0.048$; predicted means: Hill Grazing = 0.53 mmol/l, Park Grazing = 0.61 mmol/l) in the LMM analysis. Litter birth weight was positively associated with BOHB concentration. When the interaction of genetic line and management system was accounted for in the LMM model, it did not affect BOHB concentration ($P=0.248$), after adjusting for ewe weight ($P=0.646$) and ewe BCS ($P=0.052$) at scanning, first winter feeding level ($P=0.234$), number of days between sampling and lambing ($P=0.059$) and litter birth weight ($P=0.027$; positive association). Among those ewes that lambed 25 to 46 days after blood sampling (same statement for the rest of the pre-lambing metabolic profile results), only one UBF ewe (0.90 mmol/l) in 2016 had a higher BOHB concentration than the reference range, whereas 19 ewes (five UBF, nine IBF and five Lleyn; ranged from 0.82 to 1.53 mmol/l) in 2017 had higher BOHB concentrations than the reference range.

Table 3-6. Metabolite concentration values (least squares mean ± se) for UBF, IBF and Lleyn twin-bearing ewes. Means in the same row with different superscript letters were different (P<0.05).

Metabolite	Reference range	UBF	IBF	Lleyn
BOHB (mmol/l)	<0.8[1]	0.62 ± 0.18[a]	0.62 ± 0.18[a]	0.48 ± 0.18[b]
Albumin (g/l)	25-35[2]	30.82 ± 1.16[a]	30.70 ± 1.15[a]	28.63 ± 1.18[b]
Urea N (mmol/l)	3-8[2]	3.29 ± 0.33[a]	3.48 ± 0.34[a]	4.37 ± 0.34[b]
Copper (µmol/l)	9.4-19.0[3]	13.58 ± 0.69[a]	13.64 ± 0.64[a]	13.93 ± 0.73[a]
Magnesium (mmol/l)	0.7-1.3[3]	0.89 ± 0.01[a]	0.97 ± 0.01[b]	1.04 ± 0.02[c]

[1]Sargison, (2007); [2] Kerr, (2002); [3] Dairy Herd Health and Productivity Service, (2001).

The albumin concentrations of UBF and IBF twin-bearing ewes were higher than those of Lleyn twin-bearing ewes ($P<0.01$ and $P<0.001$, respectively) but the concentration of this metabolite did not differ between UBF and IBF twin-bearing ewes ($P>0.05$), after adjusting for ewe age ($P=0.272$), ewe weight ($P=0.059$) and ewe BCS ($P=0.086$) at scanning, second winter feeding level ($P=0.018$; predicted means: standard feeding = 29.47 g/l, corrective feeding = 30.64 g/l), litter birth weight ($P=0.033$) and management system ($P=0.005$; predicted means: Hill Grazing = 29.45 g/l, Park Grazing = 30.65 g/l). Litter birth weight was positively associated with the status of plasma albumin. When the interaction of genetic line and management system was fitted into the LMM model, it did not affect albumin concentration ($P=0.633$), after adjusting for ewe age ($P=0.274$), ewe weight ($P=0.077$) and ewe BCS ($P=0.109$) at scanning, second winter feeding level ($P=0.024$; predicted means: standard feeding = 29.49 g/l, corrective feeding = 30.62 g/l) and litter birth weight ($P=0.037$; positive association). Among those ewes, six ewes (two UBF and four Lleyn; ranged from 22 to 24 g/l) in 2016 had albumin concentration beneath the lower threshold of the reference range, whereas four ewes (two UBF and two IBF; ranged from 35.2 to 36.2 g/l) in 2017 had their albumin concentrations above the upper threshold of the reference range.

Lleyn twin-bearing ewes had higher urea N concentration at the blood sampling time point than their UBF and IBF counterparts ($P<0.001$ and $P<0.001$, respectively), whereas the concentration of this metabolite did not differ between UBF and IBF ewes ($P>0.05$), when the LMM model was adjusted for ewe weight at scanning ($P=0.939$) and number of days between sampling and lambing ($P=0.361$). Seven ewes (five UBF and two IBF; ranged from 2.20 to 2.90 mmol/l) in 2016 and 19 ewes (11 UBF and 8 IBF; ranged from 1.94 to 2.98 mmol/l) in 2017 had urea N concentration below the lower threshold of the reference range.

The plasma status of copper did not differ among the three twin-bearing ewe genetic lines ($P=0.919$), after ewe age ($P=0.373$), first winter feeding level ($P=0.195$) and litter birth weight ($P=0.186$) were accounted for in the LMM model. Two ewes (one UBF and one Lleyn; 4.10 and 9.30 µmol/l, respectively) in 2016 and three ewes (one UBF and two Lleyn; ranged from 7.00 to 9.20 µmol/l) in 2017 had copper concentration beneath the lower threshold of the reference range. Nine ewes (three UBF, three IBF and three Lleyn; ranged from 19.10 to 23.10 µmol/l) in 2016 and one

Lleyn ewe (20.10 µmol/l) in 2017 had copper concentration beyond the upper limit of the reference range.

Lleyn twin-bearing ewes had higher magnesium concentrations than UBF and IBF twin-bearing ewes (P<0.001 and P<0.01, respectively), and IBF twin-bearing ewes had greater magnesium concentrations than UBF twin-bearing ewes (P<0.01), after adjusting for ewe BCS at scanning (P=0.055) and management system (P=0.451). One IBF ewe (0.68 mmol/l) in 2017 had magnesium concentration below the lower threshold of the reference range, whereas another IBF ewe (1.31 mmol/l) in the same year had magnesium status just above the upper limit of the reference range.

3.4.3 Colostrum quality (lambing season 2016 and 2017)

The 10 times diluted colostrum samples of IBF twin-bearing ewes had higher Brix percentage than those of their UBF counterparts (P<0.01). However, the Brix percentage of the 10 times diluted colostrum sample did not differ either between UBF and Lleyn twin-bearing ewes, or between IBF and Lleyn twin-bearing ewes (P>0.05 and P>0.05, respectively; predicted means: UBF = 4.08%, IBF = 4.56%, Lleyn = 4.40%), when the LMM model was adjusted for ewe age (P=0.077), ewe weight at scanning (P=0.353), ewe BCS at scanning (P=0.272), first winter feeding level (P=0.035; predicted means: standard feeding = 4.41%, corrective feeding = 4.02%), litter birth weight (P=0.336) and management system (P=0.012; Hill Grazing = 4.02%, Park Grazing = 4.41%). When the interaction of genetic line and management system was considered in the LMM model, it was not a significant determinant of Brix percentages of 10 times diluted colostrum samples (P=0.384), after adjusting for ewe age (P=0.110), ewe weight (P=0.320) and ewe BCS (P=0.307) at scanning, first winter feeding level (P=0.021; predicted means: standard feeding = 4.43%, corrective feeding = 3.98%) and litter birth weight (P=0.567).

3.4.4 Post mortem examination in the 2016 and 2017 lambing seasons

Lamb mortality rates were calculated based on pure-bred lambs. The overall respective lamb mortality rates in the Kirkton flock in the 2016 and 2017 lambing seasons were 10% (60 dead lambs out of 603; 7% for UBF, 14% for IBF and 9% for Lleyn; by the end of lambing season: 30/05/2016) and 5% (40 dead lambs out of 730; 5% for UBF, 8% for IBF and 4% Lleyn; by the end of lambing season: 30/05/2017). Among the 100 dead lambs across the two lambing seasons, 25% were UBF lambs, 49% were IBF lambs, and 26% were Lleyn lambs.

Ninety-two lambs (Table 3-7 and Figure 3-13), comprising 21 UBF lambs, 47 IBF lambs and 24 Lleyn lambs were examined post mortem. The results showed that 58 lambs died as a consequence of dystocia, 20 lambs died of starvation/hypothermia, and 8 lambs died for other known reasons, with 6 deaths of unknown cause.

Table 3-7. Distribution of lamb death in each 'death category' by genetic line, gender and litter size, in 2016 and 2017 lambing seasons.

		2016				
		Dystocia	**Starvation/ Hypothermia**	**Other causes**	**Inconclusive PM**	**Total**
Genotype	**UBF**	5	6	1	0	12
	IBF	20	5	3	0	28
	Lleyn	10	2	1	0	13
Gender	**Male**	19	5	2	0	26
	Female	16	8	3	0	27
Litter size	**Single**	12	4	2	0	18
	Twin	20	9	2	0	31
	Triplet	3	0	1	0	4
		2017				
		Dystocia	**Starvation/ Hypothermia**	**Other causes**	**Inconclusive PM**	**Total**
Genotype	**UBF**	8	0	0	1	9
	IBF	12	3	1	3	19
	Lleyn	3	4	2	2	11
Gender	**Male**	16	5	3	1	25
	Female	7	2	0	5	14
Litter size	**Single**	5	3	1	1	10
	Twin	14	4	0	3	21
	Triplet	4	0	2	2	8

Dystocia accounted for 66% and 59% of lamb deaths in 2016 and 2017, respectively. Among the examined lambs, 62% of UBF lambs, 68% of IBF lambs,

and 54% of Lleyn lambs died as a consequence of dystocia. The GLMM analysis showed that lamb group (P=0.512), dam age (P=0.448), lamb birth weight (P=0.053), litter size at birth (P=0.361), lamb sex (P=0.307), lambing date (P=0.155) and ewe pre-mating BCS (P=0.669) were not significant determinants of dystocia among the lambs submitted for post mortem, whereas ewe pre-mating weight was negatively associated with the incidence of lambs dying as a consequence of dystocia (P=0.018). Lleyn lambs that died as a consequence of dystocia were heavier than their counterparts that died of starvation/hypothermia (P<0.05; predicted means: Lleyn lamb died due to dystocia = 3.84 kg, Lleyn lamb died of starvation/hypothermia = 2.77 kg). Significant differences in lamb birth weights were not found between causes of death for the UBF and IBF lambs that were post mortem examined (P>0.05 and P>0.05, respectively).

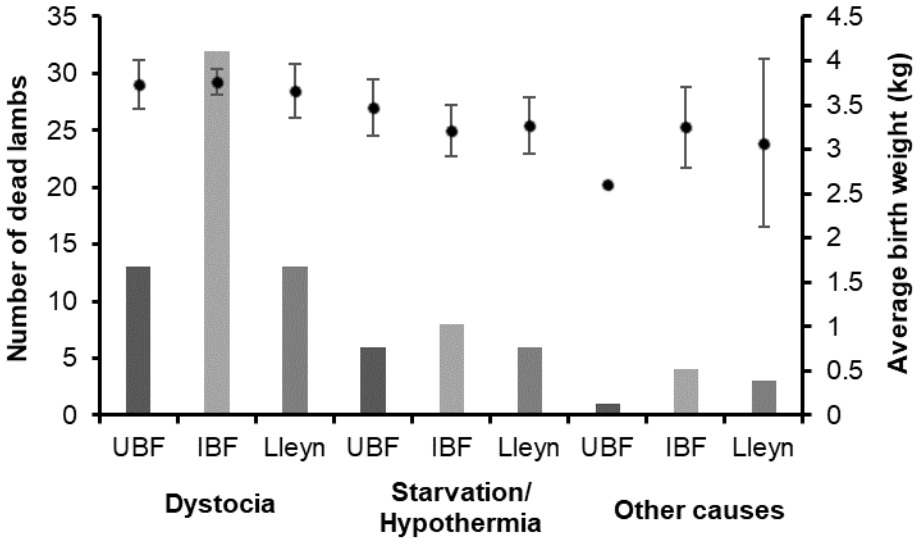


Figure 3-13. Number of dead lambs and average birth weights of the dead lambs, where cause was established, due to dystocia, starvation/hypothermia and other reasons for the three genetic lines across 2016 and 2017 lambing seasons. Bars indicate the numbers of dead lambs, whereas black dots show the mean dead lamb birth weight and vertical lines represent standard error of the mean.

3.4.5 Ewes' external pelvic width

Lleyn ewes had narrower external pelvic widths than their UBF and IBF counterparts (P<0.001 and P<0.001, respectively; predicted means: UBF = 26.4 cm, IBF = 26.4

cm, Lleyn = 25.0 cm), after adjusting for ewe age (P=0.096), ewe weight at the handling event when the pelvic width was measured (P<0.001) and management system (P<0.001; predicted means: Hill Grazing = 25.7 cm, Park Grazing = 26.3 cm). Ewe weight at the time when pelvic width was measured was positively associated with the pelvic width. When the interaction of genetic line and management system was considered in the LMM model, it did not significantly affect ewe external pelvic width (P=0.617), after adjusting for ewe age (P=0.094) and ewe weight at the handling event when the pelvic width was measured (P<0.001; positive association).

The Pearson correlation coefficient calculated between the repeated pelvic width measurements for the 48 ewes was 0.65 (P<0.001). The Pearson correlation coefficients were 0.50 (P=0.119), 0.02 (P=0.951) and 0.64 (P=0.001) for UBF, IBF and Lleyn ewes, respectively. When the difference was calculated as absolute value, between the two measurements for each individual ewe, and used as the variate in a one-way ANOVA analysis, this value did not differ significantly among the three genetic lines (P=0.288; mean absolute differences and minimum and maximum values: 1.6, 0.6 and 2.5 cm for UBF ewes; 1.4, 0.0 and 5.5 cm for IBF ewes; 1.2, 0.2 and 2.9 cm for Lleyn ewes).

Ewe external pelvic width at pre-mating was not a significant determinant of whether the ewe required assistance at lambing in the following spring (P=0.979), after the GLMM model was adjusted for genetic line (P=0.844), ewe age (P=0.413), litter birth sex (P=0.053), litter birth weight (P=0.025), ewe BCS at scanning (P=0.155) and management system (P=0.159). Litter weight at birth was positively associated with the requirement for ewe assistance at lambing. When the interaction of genetic line and management system was fitted into the GLMM model, it did not affect whether the ewe required assistance at lambing in the following spring (P=0.435), after adjusting for ewe age (P=0.380), ewe external pelvic width (P=0.946), ewe BCS at scanning (P=0.134), litter birth sex (P=0.042) and litter birth weight (P=0.038; positive association). Among single-bearing ewes, those that gave birth to male lambs were more likely to require assistance at lambing, compared to their counterparts that gave birth to female lambs (P<0.05), while no effects of litter birth sex were found among ewes that had twin and triplet litters (P>0.05 and P>0.05, respectively).

3.5 Discussion

This chapter investigated the reproductive performance of the three genetic lines (UBF, IBF and Lleyn) managed under either the Hill Grazing or the less challenging Park Grazing system. Unlike the results obtained in Chapter 2 (when ewes were managed under either the Conventional Livestock Farming system (CON) or the Precision Livestock Farming system (PLF)), litter sizes (per ewe mated) did not differ significantly among the three genetic lines, at pregnancy scanning, lambing and weaning. All three ewe genetic lines achieved better litter sizes than they had achieved in the previous study (Chapter 2; Figure 2-11), at scanning, lambing and weaning. This could be partly due to a nutritional effect on ovulation rate (Gunn et al., 1969, 1991; Robinson et al., 2002; Scaramuzzi et al., 2006). The warm and damp summer weather conditions (July and August) in 2015 and 2016 were favourable for grass growth, and thus the grass quality and availability might have been better prior to mating in those two years (Holland, unpublished), compared to the same times of year in 2012 to 2014 (Appendix 3-2 and Appendix 3-3). The grassland was also better managed compared to previous years, with grass heights measured systematically before moving sheep from one pasture to another. Additionally, the reduction of flock size from 900 ewes to 600 ewes would have resulted in a lower stocking density with better grass availability. Contrary to the scenarios demonstrated by Griffiths et al. (1970) and Doney et al. (1976), the heavy rainfall in November and December 2015 (437.2 and 738.4 mm, respectively; Appendix 3-2) did not appear to affect the overall litter size at pregnancy scanning for all three genetic lines. This might be caused by the relative higher temperature in November and December 2015 compared to previous years (average respective maximum and minimum temperatures of November and December: 9.05 and 8.97°C and 3.45 and 2.50°C in 2015 *vs.* 7.77 and 5.08°C and 1.59 and -1.04°C in 2012, 7.33 and 8.08°C and -0.51 and 2.85°C in 2013, and 9.5 and 6.04°C and 3.23 and -0.36°C in 2014; Appendix 3-3), which could have offset the detrimental effect of the heavy rainfall.

In terms of litter size at lambing (number of lambs per ewe mated), all three genetic lines performed better than BF ewes reported previously (99 lambs born per 100 ewes; Morgan-Davies et al., 2008). Additionally, ewes that were managed under the

Hill Grazing system had significantly less lambs born per ewe mated than their counterparts managed under the Park Grazing system (1.15 *vs.* 1.26). This outcome could be the consequence of both climatic and nutritional stresses (Griffiths et al., 1970; Robinson, 1990; Robinson et al., 2002). However, the litter sizes of ewes at scanning, lambing and weaning were not significantly affected by the interaction of genetic line and management system. At weaning, when excluding barren ewes, Lleyn ewes weaned more lambs than both UBF and IBF ewes, which suggests that the rearing ability of Lleyn ewes across the hill systems tested here (Hill Grazing and Park Grazing) was better than, or at least comparable with, that of UBF and IBF ewes. The positive associations between ewe pre-mating weight and litter size at pregnancy scanning (Nottle et al., 1997), and subsequently at lambing and weaning, are consistent with the concept that ewe pre-mating weight is an important determinant of ovulation rate in ewes, as it reflects long-term nutritional level, animal health conditions and other variables (e.g. age) prior to mating (Morley et al., 1978). Ewe pre-mating weight was adjusted for in the statistical models applied for litter size at pregnancy scanning, lambing and weaning. This may have eliminated the overall differences in litter size observed between the IBF ewes (which were 3.5-3.9 kg heavier on average at pre-mating than those of the other two lines; Table 3-4) and UBF or Lleyn ewes. Furthermore, the effects of the first winter feeding level on litter sizes at scanning, lambing and weaning were in agreement with the results obtained in Chapter 2. Ewes that received the standard feeding level in the first winter feeding period had had better body condition, and thus they had higher litter sizes at scanning, lambing (Gunn et al., 1969) and weaning (Donald & Russell, 1970), than their counterparts that received the corrective feeding level in the same period.

The patterns for litter birth weight per ewe lambed and average lamb birth weight (averaged across both systems) among the three genetic lines were similar to results shown in Chapter 2 (Figure 2-12), with Lleyn ewes having the heaviest lamb birth weight and litter birth weight among the three genetic lines, followed by IBF and UBF ewes. Moreover, within each genotype, litter birth weight and average lamb birth weight (averaged across both systems) in this two-year study were heavier than corresponding data reported in Chapter 2 (5.3 and 3.3 *vs.* 5.1 and 2.7 kg for UBF ewes, 5.7 and 3.5 *vs.* 5.3 and 2.8 kg for IBF ewes, 6.4 and 3.7 *vs.* 5.6 and 3.2 kg for Lleyn ewes). This could be partly due to the genetic selection for increasing

lamb weaning weights in the study flock (McLaren et al., 2012; Ceyhan et al., 2015), or simply due to other variations, such as differences in environmental conditions across years, reduction of flock size, or differences in animal health. Lamb birth weight is also an important factor for lamb survival (Dwyer & Morgan, 2006; Dwyer et al., 2016), and has a U-shaped effect on lamb mortality, with lambs at birth weighing between 3.0 and 5.0 kg having lower mortality rate than lighter or heavier lambs (Dwyer et al., 2016). In these two consecutive lambing seasons, the majority of lambs (82%) had birth weights within that optimum weight range for lamb survival (Sawalha et al., 2007; Dwyer et al., 2016). This perhaps partly explains why lower lamb mortality rates were achieved in those two years (10% and 5% in 2016 and 2017 lambing season, respectively; 12% in 2015 lambing season; mean mortality rate in the UK:10.0%; Binns et al., 2002; BF lamb mortality rate: 18%; Morgan-Davies et al., 2008).

In both lambing seasons, dystocia remained the main cause of neonatal lamb death in the flock, with more than half of the lamb deaths falling into this category. One reason could be that the weather conditions during these two lambing seasons were relatively good, and the flock was closely managed during lambing in the in-bye fields. This would have helped avoid having lambs dying from exposure to cold and wet environments or hunger. Another reason might be that ewes exposed to cold and wet weather conditions in late pregnancy had increased amounts of brown adipose tissue (Alexander, 1978; Symonds & Lomax, 1992; Symonds et al., 1992), and thus their lambs had good capacity for non-shivering thermogenesis to maintain body temperature after birth (Stott & Slee, 1985). A recent study showed that lambs born to ewes shorn in late pregnancy could maintain body surface temperature (measured using an infrared thermography camera) better, but not the core temperature (measured using a digital rectal thermometer), compared to the lambs born to unshorn ewes, during one hour exposure in 4°C environment (Labeur et al., 2017). The scenario indicated that there could be different thermoregulation mechanisms (Labeur et al., 2017). In addition, Kerslake et al. (2010) suggested that optimising lamb birth weight is important for ensuring thermoregulatory capabilities for lamb survival, something which, on the basis of 82% of lambs being within the aforementioned optimal range, had been achieved in the current study flock. Optimising lamb birth weights is likely to have enhanced neonatal lamb survival in the Kirkton flock.

In terms of lamb growth rate, the results averaged across both management systems showed that Lleyn and IBF lambs had significantly higher growth rates than UBF lambs between birth and marking. These indices of Lleyn and IBF lambs were better than that of BF lambs reported by Speijers et al. (2010; 243 g/day). This outcome was due to the much higher growth rates of Lleyn and IBF farmed under the Park Grazing sector comparing to that of their UBF counterparts (Figure 3-11). Such results suggest that under the Park Grazing system, Lleyn and IBF ewes might produce more or better quality milk in the early postnatal stage, compared to UBF ewes (Wallace, 1948; Snowder & Glimp, 1991; Treacher & Caja, 2002). Additionally, early postnatal lamb growth rates (between birth and marking; averaged across both systems) in 2016 and 2017 were higher than achieved in Chapter 2. The early postnatal lamb growth varying from year to year reflected the fact that milk secretion in the ewe is influenced by many factors, including ewe breed, nutrition, litter size and environment (Peart, 1968; Peart et al., 1979; Doney et al., 1983; Snowder & Glimp, 1991). Other factors, such as birth weight (Greenwood et al., 1998), lamb vigour, genetic line (Annett et al., 2011b), environmental conditions (Macfarlane et al., 2004) and the bond between ewe and lamb could also have effects on lamb growth during that period of time.

Lamb growth rate (averaged across both management systems) varied between marking and weaning among the three genetic lines investigated, with Lleyn lambs having the lowest growth rate during that period, which was caused by the lowest growth rate being found for Lleyn lambs among those lambs managed under the Hill Grazing system (Figure 3-12). However, they had the heaviest average lamb birth weight (averaged across both systems) and shared with IBF the higher growth rate between birth and marking (averaged across both systems). Lleyn lambs also achieved average weaning weight (averaged across both systems) heavier than or comparable to UBF and IBF lambs, and therefore Lleyn ewes achieved heavier weaned litter weight per ewe that lambed than both UBF and IBF ewes.

The higher growth rate of male lambs compared to female lambs (Bermejo et al., 2010; Bianchi et al., 2016), and the positive association between birth weight and lamb growth rate (Greenwood et al., 1998) were in agreement with the results reported in Chapter 2. Furthermore, there were not many triplets presented in the

studies in Chapter 2 and this chapter (triplets/total lambs: Chapter 2 = 65/2287, this chapter = 67/1260). If excluding triplets, the lamb growth rates (averaged across both systems) of singletons and twins between birth and marking or between marking and weaning in 2016 and 2017 followed similar trends to those of corresponding results displayed in Chapter 2 (compare Figure 3-10 with Figure 2-15). Note that the litter size at birth and at weaning were considered in the relevant statistical models applied for lamb growth rate between birth and marking or between marking and weaning. Therefore, these consistent outcomes suggest that keeping twin-bearing ewes on in-bye fields prior to and during lambing, as well as lamb birth weight (Greenwood et al., 1998) and compensatory growth in twins (Wilson & Osbourn, 1960), might all have had major influences on the differences in growth rate observed between singletons and twins, in the early postnatal stage (Figure 3-10).

Ewes of IBF genetic line had been selected for 16 years on a multi-trait index for improving both ewe and lamb performance, before the current study was commenced. In these two consecutive sheep production years, the results showed that barren rate and litter sizes at scanning, lambing and weaning did not differ between UBF and IBF genetic lines. This outcome suggests that genetic selection had had not improved ewe performance on these traits under such farming conditions. The litter sizes at lambing and at weaning of UBF and IBF ewes were lower than those reported for BF ewes farmed in hill systems in North Ireland (litter size at lambing: 1.17 and 1.18 *vs.* 1.44, litter size at weaning: 1.02 and 0.99 *vs.* 1.17, Speijers et al., 2010; litter size at lambing: 1.17 and 1.18 *vs.* 1.40, Annett et al., 2011b), which could be mainly due to that farming conditions and nutritional levels supplied differed among these experiments. Moreover, IBF ewes had heavier litter birth weight and heavier average lamb birth weight (averaged across both systems; Figure 3-5) than UBF ewes, while these indices of UBF and IBF ewes were lighter than those reported for BF ewes previously from North Irish hill farming sector (litter birth weight: 5.31 and 5.70 *vs.* 6.10 kg; average lamb birht weight: 3.28 and 3.47 *vs.* 3.67 kg; Annett et al., 2011b). Although the litter weaning weight of IBF ewes was heavier than that of UBF ewes, however, the difference was not significant. Therefore, overall lamb output of IBF ewes would only be slightly better, or at least comparable to that of UBF ewes under the farming conditions investigated in this chapter.

For neonatal lambs, consumption of sufficient and good quality colostrum can enhance lamb survival (Dwyer et al., 2016). The colostrum quality of samples taken from twin-bearing ewes was determined as Brix percentage in both 2016 and 2017 lambing seasons. This measures the percentage by weight of sucrose in liquids, such as juice or wine (Quigley et al., 2013) but is associated with total solid in milk (Moore et al., 2009). Brix percentage and SG value (as measured in Chapter 2) can be converted from one to the other, using the table provided by Mettler Toledo (2014). A previous study reported that Brix percentage of ewe colostrum had high correlation with total protein and gamma-globulin status as assayed using zinc sulphate turbidity test (0.98 and 0.79, respectively; Harker, 1978). For bovine colostrum, a Brix percentage above 22% (Bielmann et al., 2010) or 21% (Quigley et al., 2013) indicates good colostrum quality. Brix percentage of 22% is equivalent to 50 mg/ml of IgG in bovine colostrum (Heinrichs, 2016). Torres-Rovira et al, (2017) suggested that Brix percentage above 21-22% indicated high quality colostrum for dairy Lacaune ewes. If the cut-off of 22% is applicable for the ewes investigated in the current study, all the colostrum samples collected within 6 hours post lambing would be classified as good quality. Moreover, 70% of Brix percentages of undiluted ewe colostrum samples (within 6 hours post lambing) were higher than the upper limit of the instrument used (>32%), thus ewe colostrum samples were 10 times diluted in order to obtain measurements within the range of the instrument. For future studies, Digital PAL-Colostrum refractometer (Brix range: 0.0 to 53.0%) would be a better option for determination of colostrum quality of ewes (ATAGO, 2019; Belkasmi et al., 2019).

The concentration of immunoglobulin in ewe colostrum decreases rapidly after lambing. A study showed that immunoglobulin concentration (mean ± SD) declined from 202.7 ± 47.7, 201.2 ± 166.3 and 118.1 ± 27.7 g/l at the first feeding (1 or 2 hours after lambing) to 103.0 ± 70.3, 77.9 ± 44.0 and 71.2 ± 26.4 g/l at the second feeding (6 hours after the first feeding), for single-, twin- and triplet-bearing ewes, respectively (Shubber et al., 1979). Another study reported that IgG (the primary immunoglobulin in sheep colostrum; Al-Sabbagh, 2009) concentration in ewe colostrum was reduced by 30% by 6 hours after lambing (Chniter et al., 2016). Therefore, the study of colostrum quality in this chapter focused on the samples collected within 6 hours post lambing to avoid the effect of further increasing the

interval between lambing and sample collection. The results suggested that IBF ewes produced better quality colostrum than UBF ewes, but no significant difference was found between UBF and Lleyn or IBF and Lleyn ewes. Contrary to the results published by Torres-Rovira et al. (2017; Lacaune ewe), ewe age did not significantly affect Brix percentages of the colostrum samples collected within 6 hours post lambing. Furthermore, the first winter feeding level which was provided based on individual ewe live weight and BCS, had significant impact on colostrum quality, with ewes that had better body conditions in early pregnancy having higher Brix percentage colostrum after lambing than those that had poorer body conditions. That reflects the effects of nutrition and ewe BCS on colostrum production (Al-Sabbagh, 2009). In future studies, concentration of IgG could be measured using a sheep IgG ELISA kit (Genway Biotech Inc; Belanche et al., 2019). Thus, the association between refractive index and IgG content in the colostrum would be able to be detected, for the sheep breeds investigated in this study. Moreover, fat in colostrum is an important energy source for maintaining neonatal metabolism (Dwyer & Lawrence, 2005), and previous studies showed that colostral fat content of hill sheep breeds (e.g. BF) is higher, compared to that of lowland sheep breeds (e.g. Suffolk; Dwyer & Lawrence, 2005; Dwyer & Morgan, 2006). Therefore, determination of IgG, total protein and fat contents of the colostrum samples would help to detect the associations between these parameters and refractive index obtained using refractometry. This could in turn provide more cogent criteria for estimating colostrum quality using a refractometer.

Pre-lambing metabolic profiling was performed to determine twin-bearing ewes' nutritional status in late pregnancy (O'Doherty & Crosby, 1998; Antunovic et al., 2011). The same 'window' between blood sampling and lambing as used in Chapter 2 (25 to 46 days) was applied in the current study, in order to obtain comparable data. Over the two-year study, the mean concentrations of BOHB for all three genetic lines of twin-bearing ewes were within the reference range (<0.8 mmol/l), which indicated that in general, all the twin-bearing ewes were adequately nourished in late pregnancy (Sargison, 2007). The significantly higher BOHB concentrations in UBF and IBF twin-bearing ewes suggested that these twin-bearing ewes mobilised more body fat than Lleyn ewes to meet their energy requirements (Sargison, 2007). This scenario might be partly explained by UBF and IBF ewes having significantly lower magnesium concentration than Lleyn ewes. The fact that there is no storage

of magnesium in a sheep's body (Sykes, 2007; Dairy Herd Health and Productivity Service, 2014; Robinson, 2018), combined with these results, suggests that UBF and IBF twin-bearing ewes had lower feed intakes than their Lleyn counterparts. The increased appetite in Lleyn ewes might be the result of greater sensitivity to cold weather conditions in these ewes, compared to BF ewes (Young, 1983). Consequently, in comparison to Lleyn ewes, BF twin-bearing ewes had less energy supply via diet, so more body fat could have been required to be mobilised. Additionally, the breed effect on BOHB concentration discovered in this chapter was in agreement with Fogarty et al. (1992), although different ewe genotypes (Booroola Merino x Dorset, Trangie Fertility Merino x Dorset and Border Leicester x Merino) were used in their study. Therefore, the current outcome could also be, in part, due to the fact that as a hardy hill sheep breed, BF ewes are inherently more inclined to utilise body fat reserves when encountering harsh conditions, compared to lowland/upland sheep, such as Lleyns. In the 2015 study, BOHB plasma status did not differ significantly among the three twin-bearing ewe groups (Chapter 2). The contrasting outcomes in terms of the effect of genetic line on BOHB concentration reported in Chapter 2 and the current chapter confirm that BOHB status of late pregnant ewes may be influenced by many factors, such as nutrition (O'Doherty & Crosby, 1998) and rumen capacity (Sargison, 2007), and thus could vary year-by-year. In March 2017, 19 ewes had BOHB concentrations higher than the upper limit of the reference range, although for some it was only marginal and indeed none of them showed symptoms of pregnancy toxaemia around lambing time. It is acknowledged that assessing BOHB status of ewes in late pregnancy is particularly important in the prevention of pregnancy toxaemia (Sargison, 2007; Robinson, 2018).

The predicted mean concentrations of albumin and urea N were within the relevant reference ranges for all three genetic lines, which suggests that in general, the feeding provided for twin-bearing ewes was adequate for meeting their protein requirements. Plasma concentration of albumin has been demonstrated as an indicator of long-term protein intake (Caldeira et al., 2007; Robinson, 2018). The current results (see Section 3.4.2) showed that the status of this protein parameter in late pregnancy can be influenced by feeding regimens (O'Doherty & Crosby, 1998). Sykes & Thompson (1978) reported that albumin status in late pregnancy was strongly associated with calculated changes in maternal body protein content.

Low energy intake might lead to a reduction in albumin, but not in BOHB concentration. Amino acids, via gluconeogenesis, generate substrates for energy metabolism. Therefore, protein metabolism is inescapably affected by energy status, and thus the metabolism of any protein might not particularly relate to protein intake. Furthermore, unlike the results discovered in the 2015 study (Chapter 2; no significant differences in albumin and urea N concentrations among the three genetic lines), Lleyn ewes in 2016 and 2017 had significantly lower albumin concentration and significantly higher urea N concentration than UBF and IBF ewes. The latter could be due to the presumptively higher feed intake of Lleyn twin-bearing ewes, compared to their BF counterparts, as discussed previously.

In 2016 and 2017, the predicted mean concentrations of copper for the three twin-bearing ewe groups at the blood sampling time point were within the reference range. The outcome of no significant difference in this metabolite among the three genetic lines agreed with the result obtained in Chapter 2. Copper deficiency is not a major concern in the region where the study was conducted (Robinson, 2018), and none of the ewes had copper levels lower than the lower limit of the reference range in March 2016 and 2017.

In September 2016, ewe pelvic width was measured to investigate whether this index was associated with lambing difficulty during parturition. The results showed that Lleyn ewes had significantly narrower external pelvic width than UBF and IBF ewes. These results were different from the findings of no significant difference between breeds, demonstrated by Fogarty & Thompson, (1974; ewes studied: Dorset Horn and Border Leicester ewe) and Adam, (2014; ewes studied: Greyface, North of England Mule, Scotch Mule, Texel and Suffolk). The positive association between ewe pre-mating weight and ewe pelvic width agreed with the paper published by Fogarty & Thompson (1974). Adam (2014) reported that Texel ewes had significantly shorter pelvic lengths (the distance between tuber coxae and tuber ischium on the same side of a ewe) than Greyface, North of England Mule and Scotch Mule ewes. A higher incidence of dystocia in Texel ewes compared to several other breeds has been reported previously (Carson et al., 1999). This might suggest that pelvic length, not measured in the current experiment, could be a more relevant parameter for lambing difficulty, as it has a better correlation with pelvic area than pelvic width (correlation coefficients: pelvic length = 0.72, pelvic width =

0.49; Fogarty & Thompson, 1974). This also indicates that pelvic width and pelvic length should be both measured for predicting pelvic area, and these measurements probably differ among breeds (Quinlivan, 1971; Adam, 2014). The current study showed that lambing difficulty (non-assisted or assisted lambing) was affected by litter birth weight rather than by ewe pelvic width, measured in this way (Fogarty & Thompson, 1974). In this case, there might be other causes during parturition, such as foetal postural abnormality, incomplete dilation of the cervix and simultaneous presentation of lambs (Thomas, 1990), that resulted in dystocia in the current study flock.

The repeat measurements of pelvic width of 48 ewes suggested that the method used for measuring external pelvic width was not wholly reliable, especially for ewes such as IBF, as they can be very active at the handling event, making it difficult to standardise posture. In addition to this, previous publications demonstrated that internal pelvic dimension could not be accurately estimated based on external pelvic measurements (Bassett, 1955; McSporran & Fielden, 1979), and so dissection, radiography and computer tomography have been recommended for such measurements (Bassett, 1955; McSporran & Fielden, 1979; McSporran & Wyburn, 1979; Bilbe et al., 2005; Bünger et al., 2011).

Among the ewe and lamb performance traits, and those relevant causes that might lead to performance differences among the genetic lines investigated in this chapter, the interaction of genetic line and management system had significant effects on average lamb birth weight, average lamb weaning weight, lamb growth rate between birth and marking, and lamb growth rate between marking and weaning. Under the more extensive Hill Grazing conditions, both IBF and Lleyn ewes were capable to produce heavier lambs compared to their UBF counterparts, while there was no such difference between IBF and Lleyn ewes. Within each genetic line, only Lleyns farmed under the Hill Grazing system had a lighter average lamb birth weight than their counterparts farmed under the Park Grazing system. Therefore, the more extensive hill conditions featured in this study did compromise the ability of Lleyn ewes to produce heavier lambs. Nevertheless, average lamb birth weights of the six ewe subgroups (i.e. UBF, IBF and Lleyn managed under the Hill Grazing system, and UBF, IBF and Lleyn managed under the Park Grazing system) were all within the aforementioned weight range of 3.0-5.0 kg, which is important for lamb survival,

as described previously (Dwyer et al., 2016). Between birth and marking, when lamb growth mainly relies on mother's milk (Snowder & Glimp, 1991; Treacher & Caja, 2002), lamb growth rate did not differ among the three genetic lines in the more extensive hill conditions. Additionally, the growth rate of Lleyn lambs farmed under the Hill Grazing system did not differ from that of their counterparts farmed under the Park Grazing system. This scenario suggests that the capacity of milk production of Lleyn ewes farmed under the more extensive hill conditions might not be severely compromised, compared to those farmed under the Park Grazing system. From 56 days post lambing (similar to the period from marking to weaning in the current study), the dependency of lamb growth on milk converts to mainly relying on grazing and forage intake (Snowder & Glimp, 1991). During this period, growth rates of Lleyn lambs farmed under the Hill Grazing system were significantly slower than those of their UBF and IBF counterparts, while this parameter did not differ between UBF and IBF lambs farmed under the Hill Grazing system. Combining with the result of no difference in growth rate between marking and weaning being found among the three genetic lines farmed under the Park Grazing system, this outcome indicates that in harsher conditions, adaptation of foraging and/or feed conversion efficiency of Lleyn lambs might not be as good as those of UBF and IBF counterparts (McClintont & Carson, 2000, lamb breeds investigated were Greyface, Texel and Rouge; Speijers et al., 2009, lamb breeds investigated were Lleyn cross, Cheviot cross, Texel cross, Swaledale cross and BF). Consequently, within the Hill Grazing system, the average lamb weaning weight of Lleyn ewes was comparable to those of their BF flockmates. Not surprisingly, IBF ewes managed under the Hill Grazing system had heavier average lamb weaning weight than their UBF counterparts, as the result of being selected for improving lamb weaning weight for 16 years. Notwithstanding, the key production trait, weaned litter weight per ewe that lambed was not significantly affected by the interaction of genetic line and management system. Therefore, averaged across the two management systems, Lleyn ewes achieved the heaviest weaned litter weight among the three genetic lines. Notably, these system effects are likely to be cumulative across years, as ewe lambs are retained in the same system as they were born into. Thereby, further investigation under these management systems should be continued.

3.6 Conclusion

This chapter investigated the reproductive performance of the UBF, IBF and Lleyn ewes, with half of the flock being managed under the Hill Grazing system in relatively harsher conditions, compared to the Park Grazing system, under which the other half of the flock was managed. The results, averaged across grazing systems, showed that the litter sizes (number of foetuses/lambs per ewe mated) did not differ significantly among the three genetic lines, at pregnancy scanning, lambing and weaning, when adjusted for ewe live weight and other relevant terms. Among the three genetic lines, Lleyn ewes produced the heaviest litters at lambing, relative to their own weight pre-mating, and they also weaned the heaviest litters, while perhaps consuming more feed than BF flockmates, as implied by their higher magnesium status. The results (averaged across the two management systems) from this chapter confirmed the findings in Chapter 2 that Lleyn ewes may be an option for improving the productivity and profitability of hill sheep enterprises. However, Lleyn ewes farmed under the Hill Grazing system produced and weaned lighter lambs, compared to their counterparts farmed under the Park Grazing system. These effects of management systems might be cumulative, and the weather conditions between November 2015 and October 2017 were not particularly extreme. Therefore, it would be interesting to conduct further investigations under these management systems (Hill Grazing *vs.* Park Grazing).

Chapter 4: Development of a HPLC-MS/MS assay for the determination of concentrations of vitamin D metabolites in sheep serum

4.1 Summary

High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) is an accurate and precise method for quantification of vitamin D metabolites in blood samples collected from human and several animal species. The aim of the experiment was to develop a HPLC-MS/MS method to simultaneously measure the concentrations of 9 vitamin D metabolites (i.e. vitamin D_2, vitamin D_3, 25(OH)D_2, 25(OH)D_3, 1α,25-(OH)$_2D_2$, 1α,25-(OH)$_2D_3$, 3-epi-25(OH)D_2, 3-epi-25(OH)D_3 and 24R,25(OH)$_2D_3$) in sheep serum samples. This newly derived method quantified the concentrations of the key vitamin D metabolites, 25-Hydroxyvitamin D_2 (25(OH)D_2) and 25-Hydroxyvitamin D_3 (25(OH)D_3), in sheep serum samples, for further analyses relevant to the aims of this thesis. Efficiency of the method was evaluated, and the results showed that extraction recovery rates were 63% for 25(OH)D_2 and 54% for 25(OH)D_3, and injection carryover was ≤ 0.32% for both vitamin D metabolites and, in each case, the corresponding internal standard. The lower limits of quantification (LLOQ) were 7.2 nmol/l for 25(OH)D_2, and 5.6 nmol/l for 25(OH)D_3, lower than the figures reported by Wallace et al. (2010). The concentrations of 25(OH)D_2 and 25(OH)D_3 in serum samples collected from Soay sheep in North West Scotland were quantifiable using the newly derived method.

4.2 Introduction

Vitamin D has an important role in maintaining musculoskeletal health (Holick, 2007; the synthesis and metabolism of vitamin D have been described in Section 1.6.4.1). Recent studies in humans suggest that vitamin D deficiency is a risk factor for certain chronic health problems, such as autoimmune diseases, cardiovascular disorders and cancer (Lappe et al., 2007; Holick, 2007; Zhang & Naughton, 2010). This has led to growing research interest in vitamin D status in relation to animal

health and performance (Aslam et al., 1998; Coffey et al., 2012; Titmarsh et al., 2015a,b). A recent study of Soay sheep – a wild population in the St Kilda Archipelago, in North West Scotland – demonstrated that ewe reproductive performance was significantly associated with total 25(OH)D serum concentration (Handel et al., 2016). The Scottish sheep farming sector comprises more than 6.8 million animals, generating greater than £200 million in financial outputs annually (The Scottish Government, 2017). Thus, it would be of interest to develop a method to simultaneously assay the concentrations of multiple vitamin D metabolites in sera of commercially farmed sheep in Scotland, in order to further investigate some of these potential effects on economically important traits.

Several methods are available for determination of serum 25(OH)D concentration. However, the hydrophobic nature and the relatively low serum concentration of 25(OH)D (Zerwekh, 2004) make it difficult to detect accurately the level of this vitamin D metabolite using competitive immunoassays or competitive assays based on vitamin D binding proteins, as different sample preparation methods before analysis increase assay variability. The 25(OH)D values estimated from the same sample, but obtained from different laboratories, even by the same type of assay, could vary as much as four-fold (Carter et al., 2004). Moreover, the primary antibody used in the radioimmunoassay may not equally recognize $25(OH)D_2$ and $25(OH)D_3$, and results in underestimation of total circulating 25(OH)D (Hollis, 2000). Understanding the levels and effects of these different forms in commercial sheep would be important to help recognise and mitigate any limiting effects on health or performance.

High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) has been commonly used in quantification of vitamin D metabolites in human samples, because the technique provides high efficiency in terms of compound separation and powerful sensitivity and selectivity in analyte detection (Hansen and Reubsaet, 2015; van den Ouweland, Vogeser and Bächer, 2013). Vitamin D metabolites have low ionization efficiencies, and in order to improve the sensitivity of determination, derivatization with Cookson-type reagent, such as 4-[2-(3,4-Dihydro-6,7-dimethoxy-4-methyl-3-oxo-2-quinoxalinyl)ethyl]-3H-1,2,4-triazole-3,5(4H)-dione (DMEQ-TAD) has been employed to increase ionization efficiency (Higashi et al., 2001; Kaufmann et al., 2014).

The experiment described in this chapter developed and evaluated a HPLC-MS/MS method, involving derivatisation with DMEQ-TAD, to simultaneously determine 9 vitamin D metabolites, i.e. vitamin D_2, vitamin D_3, 25(OH)D_2, 25(OH)D_3, 1α,25-(OH)$_2D_2$, 1α,25-(OH)$_2D_3$, 3-epi-25-Hydroxyvitamin D_2 (3-epi-25(OH)D_2), 3-epi-25-Hydroxyvitamin D_3 (3-epi-25(OH)D_3) and 24R,25(OH)$_2D_3$ in sheep serum samples.

4.3 Materials and Methods

4.3.1 Chemicals and reagents

Sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), sodium hydroxide (NaOH), ammonium formate, methanol (LC-MS grade), ethanol (HPLC grade) and ethyl acetate (HPLC grade) were purchased from Fisher Scientific (Loughborough, UK). Hexane (HPLC grade), potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4) and bovine serum albumin were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Acetonitrile (HPLC LC-MS grade) and HPLC grade water were purchased from VWR International Ltd (Lutterworth, Leicestershire, UK). The derivatization reagent DMEQ-TAD was purchased from Abcam (Cambridge, UK).

Vitamin D_2 solution (1.0 mg/ml in ethanol), vitamin D_3 solution (1.0 mg/ml in ethanol), 25(OH)D_2 solution (50 µg/ml in ethanol), 25(OH)D_3 solution (100 µg/ml in ethanol), 1α,25-(OH)$_2D_2$ solution (50 µg/ml in ethanol), 1α,25-(OH)$_2D_3$ solution (5 µg/ml in ethanol), 3-epi-25(OH)D_2 solution (50 µg/ml in ethanol), 3-epi-25(OH)D_3 solution (50 µg/ml in ethanol) and 24R,25(OH)$_2D_3$ (50 µg) were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Distilled water was prepared in-house. Fifty micrograms of 24R,25(OH)$_2D_3$ were dissolved in 1 ml of ethanol. All the standard solutions were aliquoted into 10 µl for storage. The aliquots of 25(OH)D_2, 25(OH)D_3, 1α,25-(OH)$_2D_2$ and 3-epi-25(OH)D_3 stock solution were stored at -80°C, while the aliquots of the remaining stock solutions were stored at -20°C.

Internal standards (ISs), which were isotopically labelled 6,19,19-d_3-25(OH)D_2 solution (5 µg/ml in ethanol) and 23,24,25,26,27-$^{13}C_5$-25(OH)D_3 solution (100 µg/ml in ethanol), were purchased from Sigma-Aldrich (Gillingham, Dorset, UK).

4.3.2 Sheep serum samples

Soay sheep serum samples (16 samples; contributed by Prof Richard Mellanby, University of Edinburgh) were collected in August 2012 from feral animals in the Village Bay area on the island of Hirta in the St Kilda archipelago (58°N, 8°W), and they had been analysed previously for 25(OH)D_2 and 25(OH)D_3 serum concentrations in the Supra Regional Assay Service Laboratory, Manchester (Handel et al., 2016). These samples were used to assess the developed method, by comparing the results obtained from the developed method and the results of the same sample reported from the aforementioned commercial laboratory previously, at the final stage of the method development. Four BF sheep serum samples (2 UBF and 2 IBF) were collected from the Kirkton flock in mid-November 2015. These samples were used to determine inter-assay coefficient of variation (CV) of the developed method.

4.3.3 HPLC and tandem mass spectrometry (MS/MS) conditions

The instruments used for the analysis were an UltiMate 3000 HPLC system interfaced to an amaZon ETD tandem mass spectrometer (Bruker Daltonics, Bremen, Germany). Separation of vitamin D metabolites on reverse phase HPLC was achieved using an ACE UltraCore 2.5 SuperC18 column (75 x 2.1 mm id, 2.5 µm; Advanced Chromatography Technologies Ltd, Aberdeen, Scotland). The column was maintained at 40°C during analysis. The injection volume was 5 µl, and the flow rate was constant at 200 µl/min. Mobile phase A consisted of 10 mM ammonium formate with 0.15% formic acid. Mobile phase B consisted of methanol with 0.1% formic acid. The program of the initial mobile phase started with 20% of B; then there was a linear increase to 72% of B during the first minute; after maintaining at 72% of B for 6.5 min, it increased linearly to 100% of B in 0.5 min, and remained at 100% of B for 1 min; then returned to 20% of B in 0.1 min; and remained at 20% of B for 0.9 min. The total analysis time was 10 min per sample.

The elution was detected using MS/MS in positive electrospray multiple reaction monitoring (MRM) mode, with 1.0 m/z for isolation window width, 8,100 m/z/sec for scanning rate, and 4,500 v for capillary current, for all the analytes. The nebulizer pressure was 16.0 psi. The dry gas flow rate was 8.0 L/min with temperature of 220°C.

4.3.4 Derivatisation with DMEQ-TAD reagent

Derivatization with DMEQ-TAD reagent was via the method published by Kaufmann et al. (2014). Briefly, a sample or calibration standard was derivatised with 25 µl of 0.1 mg/ml DMEQ-TAD in ethyl acetate by incubation for 30 min at room temperature in the dark. Another 25 µl of 0.1 mg/ml DMEQ-TAD in ethyl acetate was added and the mixture was further incubated for 1 hour at room temperature in the dark. After addition of 40 µl of ethanol, the mixture was evaporated to dryness at 45°C in the SAVANT SPD 2010 SpeedVac Concentrator (Thermo Scientific, Loughborough, UK) for 1 h.

4.3.4.1 Comparison of derivatised and underivatised vitamin D_2 and D_3

Two microlitres of vitamin D_2 or 2 µl of vitamin D_3 stock solution were transferred into a new 1.5 ml micro-tube. Each of these underivatised stock solutions was diluted with 998 µl of 60:40 (vol:vol) methanol with 0.1% formic acid:water.

Two microlitres of vitamin D_2 or 2 µl of vitamin D_3 stock solution were transferred into a new 1.5 ml micro-tube, and derivatised with DMEQ-TAD reagent. The dried stock solution was reconstituted in 1 ml of 60:40 (vol:vol) methanol with 0.1% formic acid:water.

The MRM transition, fragmentation cut-off and amplitude of underivatised and derivatised vitamin D_2 and D_3 were detected using an amaZon ETD tandem mass spectrometer by a direct infusion method with a syringe pump. The flow rate was 2 µl/min.

4.3.5 Determination of the optimised fragmentation conditions and retention time

Each vitamin D metabolite stock solution (2 µl) was transferred into a 1.5 ml micro-tube for derivatisation with DMEQ-TAD reagent. The 9 micro-tubes containing 9 different dried derivatised vitamin D metabolites were reconstituted with 60:40 (vol:vol) methanol with 0.1% formic acid:water (see Table 4-1 for volume applied) to the required concentrations. These samples were used to detect the 1st MRM transition using the amaZon ETD mass spectrometer by a direct infusion method with a syringe pump. The flow rate was 2 µl/min. The highest intensity transition was selected to quantify the analyte with the highest sensitivity. All 9 vitamin D metabolites, except for vitamin D_2 and D_3, were detected for the 2nd mass transition by selecting the product ion of the 1st mass transition (see Table 4-3 in the results section) as the 2nd precursor to run MS/MS manual analysis with a syringe pump, and then select high intensity fragment as 'Target Mass', to determine the optimized fragmentation cut-off and amplitude for the 2nd precursor.

Table 4-1. The concentrations of vitamin D metabolites used for detecting the 1st and 2nd MRM transition.

Vitamin D metabolite	Concentration of stock solution	Stock solution (µl)	60:40 methanol &0.1% FA:water	Concentration (nmol/l)
Vitamin D_2	1 mg/ml	2	1 ml	5.0×10^3
Vitamin D_3	1 mg/ml	2	1 ml	5.2×10^3
25(OH)D_2	50 µg/ml	2	250 ul	9.6×10^2
25(OH)D_3	100 µg/ml	2	500 ul	1.0×10^3
1α,25-$(OH)_2D_2$	50 µg/ml	2	250 ul	9.3×10^2
1α,25-$(OH)_2D_3$	5 µg/ml	2	50 ul	4.8×10^2
3-epi-25(OH)D_2	50 µg/ml	2	250 ul	9.7×10^2
3-epi-25(OH)D_3	50 µg/ml	2	250 ul	1.0×10^3
24R,25$(OH)_2D_3$	50 µg/ml	2	250 ul	9.6×10^2

A mixture of 9 vitamin D metabolites was generated by transferring 5 µl of each vitamin D metabolite into a 1.5 ml micro-tube, and the mixture was derivatised with DMEQ-TAD reagent. This dried derivatised mixture was reconstituted in 50 µl of 60:40 (vol:vol) methanol with 0.1% formic acid:water. The remaining samples that were prepared for detecting mass transition (see Table 4-1), and the mixture of 9 vitamin D metabolites were used to detect the retention time of each vitamin D metabolite using the HPLC-MS/MS system with initial mobile phase gradient (detailed in Section 4.3.3).

4.3.6 Sample purification

Liquid-liquid extraction (LLE) and solid phase extraction (SPE) for sample purification were both tested, and the sample recovery rate was used to finalise the sample purification method. The methods reported for protein precipitation, LLE and SPE in scientific publications were reviewed and are listed in Appendix 4-1 and Appendix 4-2.

Two samples were prepared by addition of 2 µl of vitamin D_2 stock solution or 2 µl of vitamin D_3 stock solution into a micro-tube containing 100 µl of artificial serum (prepared in-house by dissolving 50 mg of bovine serum albumin into 1 ml of phosphate buffered saline with a final concentration of 0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4). Another 2 samples were prepared by addition of 2 µl of 1 in 10 diluted vitamin D_2 stock solution or 2 µl of 1 in 10 diluted vitamin D_3 stock solution into a micro-tube containing 100 µl of artificial serum. These 4 samples were protein precipitated with acetonitrile (Section 4.3.6.1), and then extracted through either LLE (Section 4.3.6.2) or SPE (Section 4.3.6.3) for each concentration.

4.3.6.1 Protein precipitation

After equilibration at room temperature for 15 minutes, each sample was protein precipitated by addition of 200 µl of acetonitrile (Vogeser et al., 2004; Aronov et al., 2008) and incubated for 10 minutes, before centrifugation at 15,700 *g* for 10 min. The supernatant was transferred to a clean 1.5 ml micro-tube.

4.3.6.2 LLE procedure

Four hundred microlitres of hexane was added into the supernatant obtained from the protein precipitation (Maunsell et al., 2005). The contents of the micro-tube were mixed for 1 minute using a vortex mixer, followed by centrifugation at 10,000 *g* for

10 minutes. The hexane layer was removed to a clean micro-tube. The organic solvent was evaporated to dryness using nitrogen in a fume hood.

4.3.6.3 SPE procedure

The method used for SPE of vitamin D metabolites was a modification of that reported by Aronov et al (2008). An Oasis HLB extraction cartridge (1cc, 10 mg sorbent; Waters) was activated with 3 ml of ethyl acetate, 3 ml of methanol, and 3 ml of distilled water. The next solution was added into the cartridge after the water meniscus reached the sorbent surface. The supernatant from protein precipitation was diluted with 1 ml of 0.4 M K_2HPO_4. The diluted supernatant was loaded into the cartridge in two steps, and a sample was extracted using gravity only. The cartridge was subsequently washed with 3 ml of distilled water followed by 2 ml of 40% methanol, and dried for 2 minutes with application of positive pressure using a pipette. Vitamin D metabolites were eluted with 1.5 ml of acetonitrile. The collected samples were evaporated to dryness using nitrogen in the fume hood.

The 4 extracted samples were derivatised with DMEQ-TAD reagent. Two control samples were prepared accordingly to contain the same concentrations of vitamin D_2 and D_3, as the samples had gone through extraction procedures, and they were directly derivatised with DMEQ-TAD reagent. The dried derivatised controls and samples were reconstituted in 1 ml of 60:40 (vol:vol) methanol with 0.1% formic acid:water. After vigorous mixing, each sample was transferred into a 300 µl glass vial (Thermo Scientific; Loughborough, UK) for HPLC-MS/MS analysis using the initial mobile phase gradient. The sample recovery rate was calculated by comparing the total peak areas of the extracted samples to those of the corresponding control samples.

4.3.7 Analysis of Soay sheep samples

4.3.7.1 Sample preparation and HPLC-MS/MS method

Sixteen Soay sheep serum samples were analysed in three runs using the developed method. Eight calibration standards were freshly prepared for each run. The concentrations of vitamin D metabolites for each calibration standard are summarised in Table 4-2.

Table 4-2. The concentrations (nmol/l) of the 9 vitamin D metabolites in calibration standards.

Vitamin D metabolite	Calibration standard (nmol/l)							
	1st	2nd	3rd	4th	5th	6th	7th	8th
Vitamin D_2	603.10	201.03	67.01	22.34	7.45	2.48	0.83	0.28
Vitamin D_3	622.00	207.33	69.11	23.04	7.68	2.56	0.85	0.28
25(OH)D_2	347.90	115.97	38.66	12.89	4.30	1.43	0.48	0.16
25(OH)D_3	955.40	318.47	106.16	35.39	11.80	3.93	1.31	0.44
1α,25-$(OH)_2D_2$	223.20	74.40	24.80	8.27	2.76	0.92	0.31	0.10
1α,25-$(OH)_2D_3$	344.50	114.83	38.28	12.76	4.25	1.42	0.47	0.16
3-epi-25(OH)D_2	116.00	38.67	12.89	4.30	1.43	0.48	0.16	0.05
3-epi-25(OH)D_3	119.40	39.80	13.27	4.42	1.47	0.49	0.16	0.05
24,25$(OH)_2D_3$	229.70	76.57	25.52	8.51	2.84	0.95	0.32	0.11

After each sheep serum sample was thawed at room temperature and vigorously agitated, 100 µl of calibration standard or sheep serum sample was spiked with 2 µl of 6,19,19-d_3-25(OH)D_2 (diluted to 1.78 µmol/l) and 2 µl of 23,24,25,26,27-$^{13}C_5$-25(OH)D_3 (diluted to 2.47 µmol/l). After equilibration at room temperature for 15 minutes, 20 µl of NaOH was added to release protein-bound analytes and the ISs during a 20-minute incubation period (Vogeser et al., 2004). The calibration standard or sheep sample was then protein precipitated by addition of 200 µl of acetonitrile (see Section 4.3.6.1).

Soay sheep serum samples or calibration standards were purified using a modified version of the SPE described in Section 4.3.6.3. A Discovery DSC-18 SPE-96 Plate (bed wt: 25 mg/well; Sigma-Aldrich) was activated with 3 ml of ethyl acetate and 3 ml of methanol, followed by equilibration with 3 ml of distilled water. After each 1 ml addition, the plate was centrifuged in a KR 4i centrifuge (Thermo Scientific, Loughborough, UK) at 18°C, 100 *g* (settings for temperature and relative centrifugal

force were the same in subsequent SPE programmes) for 2 minutes. The supernatant from protein precipitation (approximately 300 µl) was diluted with 1 ml of 0.4 M K_2HPO_4, and then loaded into the plate well. The 1st loading (1 ml) was centrifuged for 2 minutes, the 2nd loading (approximately 300 µl) was centrifuged for 1 minute. The plate wells were subsequently washed with 3 ml of distilled water followed by 2 ml of 40% methanol. During washing, the plate was centrifuged for 2 minutes for each 1 ml addition of water; the plate was then centrifuged for 2 minutes for the 1st addition of 1 ml 40% methanol, and 3 minutes for the 2nd addition of 1 ml 40% methanol. After centrifugation, the residual droplets at the end of the plate well were wiped off using a clean tissue. Vitamin D metabolites were eluted with 1.5 ml of acetonitrile into a clean collection plate (2 ml per well; Sigma-Aldrich), with centrifugations lasting 2 minutes for both the 1 ml and 0.5 ml additions of acetonitrile. The collected samples were transferred into clean micro-tubes, and they were submitted for evaporation at 45°C in a SAVANT SPD 2010 SpeedVac Concentrator for 1.5 h.

After derivatisation with DMEQ-TAD, the dried derivatised extract was reconstituted in 50 µl (1st run) or 25 µl (2nd and 3rd run) of 60:40 (vol:vol) methanol with 0.1% formic acid:water. After vigorous mixing, the sample solution was transferred into a 300 µl glass vial for HPLC-MS/MS analysis using the initial mobile phase gradient (1st run) or the revised mobile phase gradient (2nd and 3rd run; detailed in Section 4.3.7.2).

Quantitation was carried out using Bruker Compass QuantAnalysis 2.0 SP 2 (Bruker Daltonik GmbH, Bremen, Germany) software. A standard curve was generated for each analyte, based on the ratio of the larger peak area of the standard to that of the corresponding IS.

4.3.7.2 Method modification

Determination of the concentrations of vitamin D_2 and D_3 was unsuccessful from the analysis based on the results of the 1st run of Soay sheep samples, and consequently the mobile phase gradient was revised to improve the separation of the other 7 vitamin D metabolites during the elution time. The revised mobile phase

gradient (in contrast to that in Section 4.3.3) started with 20% of B; then a linear increase to 72% of B in 1 min; after maintaining at 72% of B for 6.5 min, it further increased to 100% of B in 1.5 min (this 90 s step being the sole modification); then returned to 20% of B in 0.1 min; and remained at 20% of B for 0.9 min. The total analysis time was 10 min per sample, and the system was then re-established and equilibrated with 20% of B for 2 minutes.

Stock standards of 25(OH)D_2 (5 µg/ml in ethanol; Sigma-Aldrich) and 25(OH)D_3 (5 µg/ml in ethanol; Sigma-Aldrich) were purchased for the analysis from the 3rd run of Soay sheep samples. The concentrations of calibration standards were modified to 230.8, 115.4, 57.7, 28.9, 14.4, 7.2, 3.6 and 1.8 nmol/l for 25(OH)D_2; and 356.6, 178.3, 89.2, 44.6, 22.3, 11.2, 5.6 and 2.8 nmol/l for 25(OH)D_3.

4.3.8 Method validation

The method validation was based on the revised HPLC-MS/MS method for examining the concentration of 25(OH)D_2 and 25(OH)D_3 in sheep serum samples. Each sample or calibration standard was assayed in duplicate throughout the sample analyses. Intra-assay CV was obtained based on 5 different runs. Inter-assay CV was determined on the basis of results from four BF sheep serum samples analysed in 3 consecutive runs. Every standard that had an observed concentration within 30% of intent was considered acceptable in the current experiment (Fritz et al., 2017).

The extraction recovery during sample preparation was determined by comparison with the artificial serum spiked with 25(OH)D_2 and 25(OH)D_3 stock standards (and then put through the sample preparation procedures described in the preceding sections) with the post-extracted samples. The post-extracted samples had been generated by spiking the same amount of stock standards into a tube that contained dried artificial serum which had already undergone protein precipitation and SPE (Fritz et al., 2017). Three different concentrations (233.0, 116.5 and 58.3 nmol/l for 25(OH)D_2; and 234.0, 120.0 and 60.0 nmol/l for 25(OH)D_3) of samples were prepared in duplicate. The total peak areas were used to calculate the efficiency of

recovery, which indicated the amount of vitamin D metabolite that was not lost during the sample preparation procedures.

Injection carryover was determined in each run, via the comparisons of the peak areas for analytes and ISs (in the validation run of the highest concentration standard) with the corresponding areas in the Blank (60:40 vol:vol methanol & 0.1% formic acid:water) that was analysed immediately afterwards. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were determined by identifying the lowest standard contained within the standard curve that had minimum signal to noise ratios of 5:1 and 10:1, respectively, and for which the observed concentration was within 30% of intent (Priego-Capote et al., 2007; Fritz et al., 2017). The ratio was calculated based on the height of the major peak relative to that of the average background noise obtained in the relevant chromatograms (Priego-Capote et al., 2007).

The results for 25(OH)D_2 and 25(OH)D_3 status of Soay sheep serum samples obtained using the revised method (2nd and 3rd run) were compared with the results reported previously from an accredited lab, using Pearson correlation tests (Minitab 17 software).

4.4 Results

4.4.1 Derivatisation with DMEQ-TAD reagent

Derivatisation with DMEQ-TAD reagent could improve the sensitivity during mass spectrometry detection, as the fragment ion of DMEQ-TAD is a high molecular weight ion which provides a cleaner MRM profile during HPLC-MS/MS analysis. An example of the mass spectrum view is shown in Figure 4-1. Vitamin D_3 DMEQ-TAD derivative yielded two peaks with higher intensities and relatively lower background noise level (Figure 4-1B) compared to the same concentration of intact vitamin D_3 (Figure 4-1A).

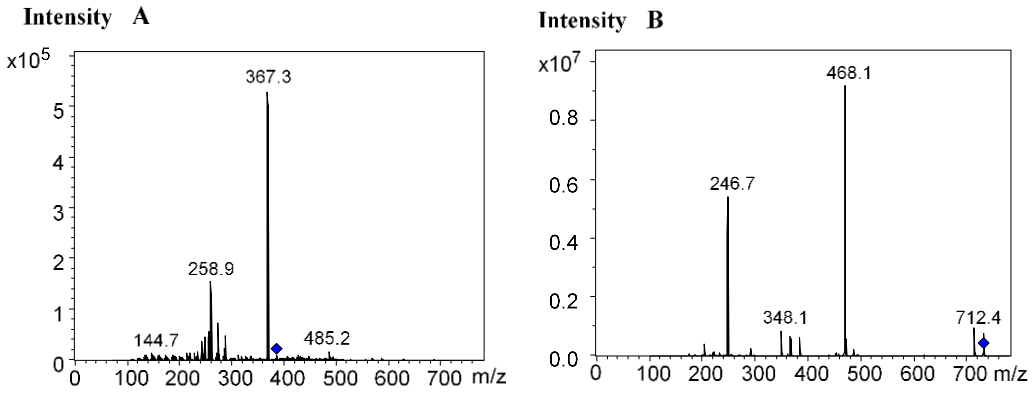


Figure 4-1. Mass spectra of intact vitamin D_3 (A) and vitamin D_3 DMEQ-TAD derivative (B).

4.4.2 Fragmentation conditions of 9 vitamin D metabolites

The transition from precursor ion to product ions of each analyte was assessed using a stock standard. Except for vitamin D_2 and D_3, loss of a water molecule occurred in the initial fragmentation for 25(OH)D_2, 25(OH)D_3, 1α,25-(OH)$_2D_2$, 1α,25-(OH)$_2D_3$, 3-epi-25(OH)D_2, 3-epi-25(OH)D_3, 24R,25(OH)$_2D_3$ (see Table 4-3), and with one mass transition, could not achieve a clean MRM profile for these 7 vitamin D metabolites. Therefore the 2nd fragmentation conditions were detected for the vitamin D metabolites listed above (see Figure 4-2 for the mass spectrum of 25(OH)D_2 with one mass transition (A) or two mass transitions (B), as an example).

Table 4-3. MRM transitions, fragmentation cut-offs, fragmentation amplitudes and retention times of the 9 vitamin D metabolites.

Vitamin D metabolite	MRM transition	Cut-off (m/z); Amplitude (v)		Retention times (min)
		1st mass transition	2nd mass transition	
Vitamin D_2	742.5>468.1	184; 0.95	-	9.5 & 9.6
Vitamin D_3	730.5>468.1	173; 0.70	-	9.5 & 9.6
25(OH)D_2	758.5>740.4>468.1	163; 0.90	167; 0.53	5.3 & 6.8
25(OH)D_3	746.5>728.4>468.1	185; 0.70	164; 0.80	4.9 & 6.1
1α,25-(OH)$_2D_2$	774.4>756.4>468.1	166; 0.52	187; 0.75	4.0 & 4.7
1α,25-(OH)$_2D_3$	762.5>744.4>468.1	172; 0.63	176; 0.64	4.3 & 5.2
3-epi-25(OH)D_2	758.5>740.4>468.1	155; 1.04	167; 0.59	5.6 & 6.4
3-epi-25(OH)D_3	746.5>728.4>468.1	168; 0.55	164; 0.70	5.1 & 5.8
24R,25(OH)$_2D_3$	762.5>744.4>468.1	164; 0.64	176; 0.52	2.8 & 3.3

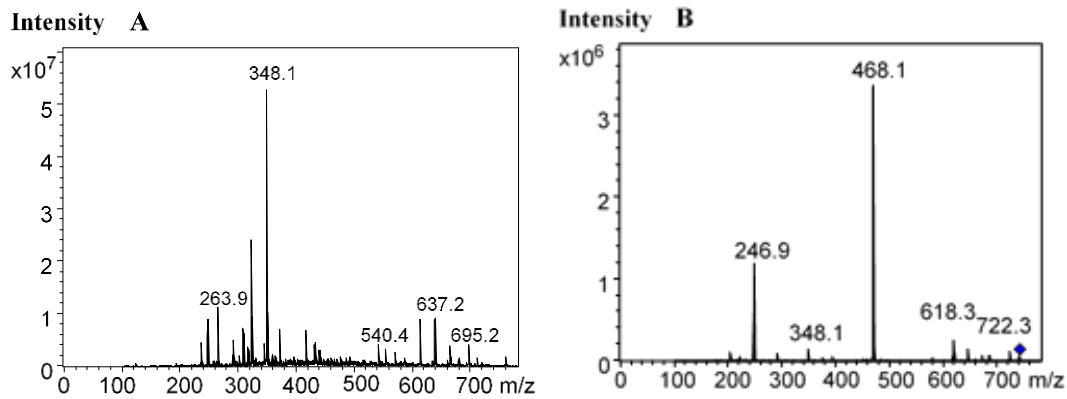


Figure 4-2. Mass spectra of DMEQ-TAD derivative of 25(OH)D_2 with one fragmentation (A) and two fragmentations (B).

The 9 vitamin D metabolites were separated during the 10 minute HPLC-MS/MS analysis using the initial mobile phase gradient (Figure 4-3). As this resultant chromatogram shows, there were two peaks for each analyte, as two isomers (6R and 6S) were formed during derivatisation with DMEQ-TAD, for each vitamin D metabolite. The MRM transition, optimised fragmentation conditions (cut-off and amplitude), and retention times of all 9 vitamin D metabolites are summarised in Table 4-3.

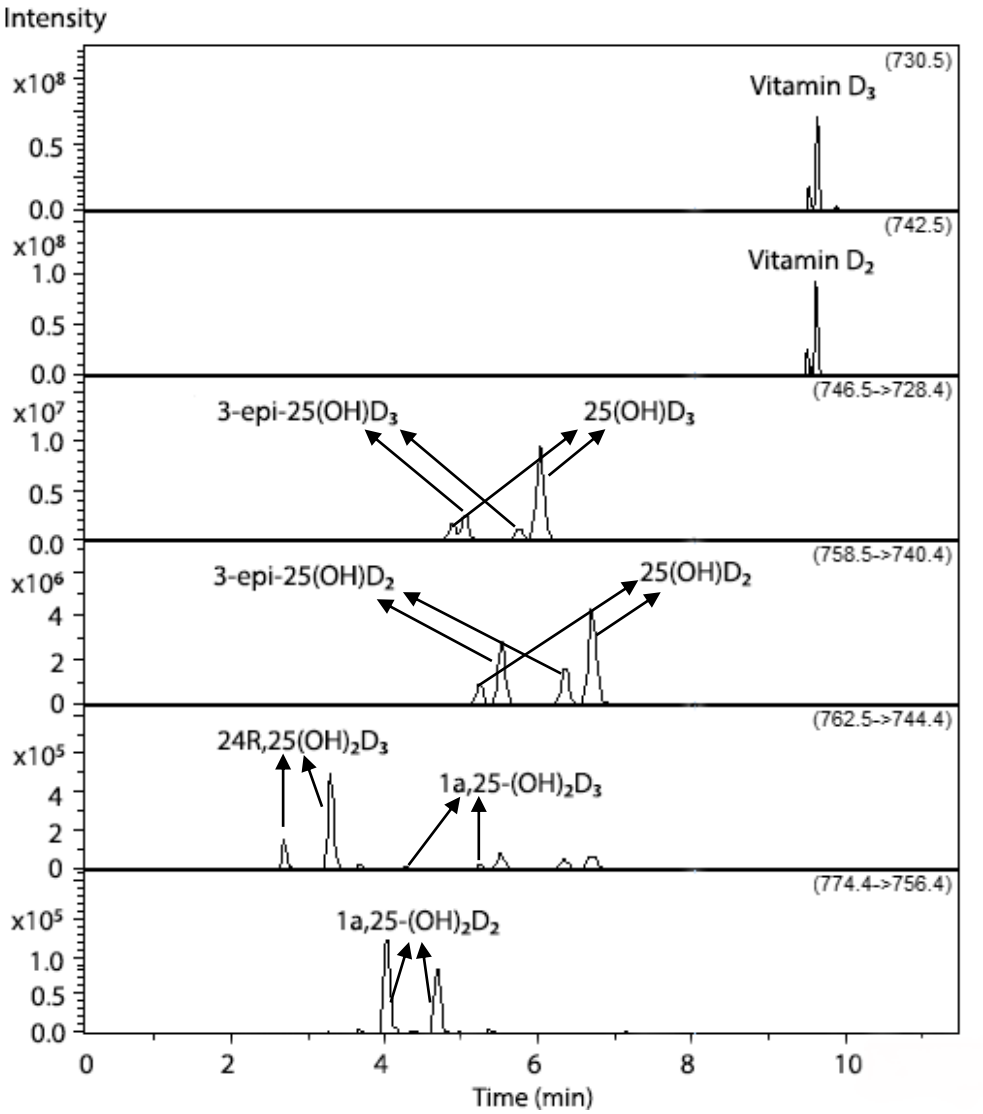


Figure 4-3. Chromatograms showing the retention times of 9 vitamin D metabolites during the 10 minute tandem mass spectrometry detection.

4.4.3 Comparison of extraction methods

LLE and SPE were compared in terms of extraction efficiency of sample preparation using vitamin D_2 and vitamin D_3 stock solutions. The total peak areas of vitamin D_2/D_3 derivatives, either through LLE or SPE, and those of controls in two different concentrations, are shown in Table 4-4. The results indicated that SPE was a more efficient method for sample purification for both vitamin D_2 and D_3, thus SPE was selected for sample preparation.

Table 4-4. Peak areas and recovery rates of the LLE and SPE extracted samples and the control samples.

Extraction method	Analyte (nmol/l)	1st peak area	2nd peak area	Total peak area	Recovery rate
LLE	D_2 (4.9 x 10^3)	4932707	22122153	27054860	27.1%
	D_3 (5.1 x 10^3)	5205310	28594277	33799587	26.9%
SPE	D_2 (4.9 x 10^3)	11717986	71222123	82940109	83.1%
	D_3 (5.1 x 10^3)	13758784	81701849	95460633	76.1%
Control 1	D_2 (4.9 x 10^3)	16621784	83158388	99780172	-
	D_3 (5.1 x 10^3)	19790780	105697111	125487891	-
LLE	D_2 (4.9 x 10^4)	50414434	222699049	273113483	44.5%
	D_3 (5.1 x 10^4)	58998920	284654050	343652970	42.0%
SPE	D_2 (4.9 x 10^4)	125996338	403772097	529768435	86.2%
	D_3 (5.1 x 10^4)	141702330	442840082	584542412	71.4%
Control 2	D_2 (4.9 x 10^4)	163482030	450796214	614278244	-
	D_3 (5.1 x 10^4)	216547507	601729302	818276809	-

4.4.4 Determination of vitamin D status in Soay sheep samples

The determination of the concentration of vitamin D metabolites in Soay sheep samples showed that the refined protocol can quantify the concentrations of 25(OH)D_2 and 25(OH)D_3 in sheep serum samples. The chromatograms of the 4th standard show that the shapes of the peaks representing vitamin D_2 and D_3 were not sharp (Figure 4-4), and there were not relevant product ions (246.7 and 468.1) yielded in the corresponding spectra (Figure 4-5; Figure 4-5A vs. Figure 4-1B for vitamin D_3 DMEQ-TAD derivative, as an example), which indicates that these two vitamin compounds were not quantifiable using the current method. Similarly, the chromatograms of Soay sheep samples suggested that 1α,25-$(OH)_2D_2$, 1α,25-$(OH)_2D_3$, 3-epi-25(OH)D_2, 3-epi-25(OH)D_3 and 24R,25$(OH)_2D_3$ could not be quantified using this method, due to the low intensities of the corresponding peaks and high background noise levels, as shown in Figure 4-6 as an example.

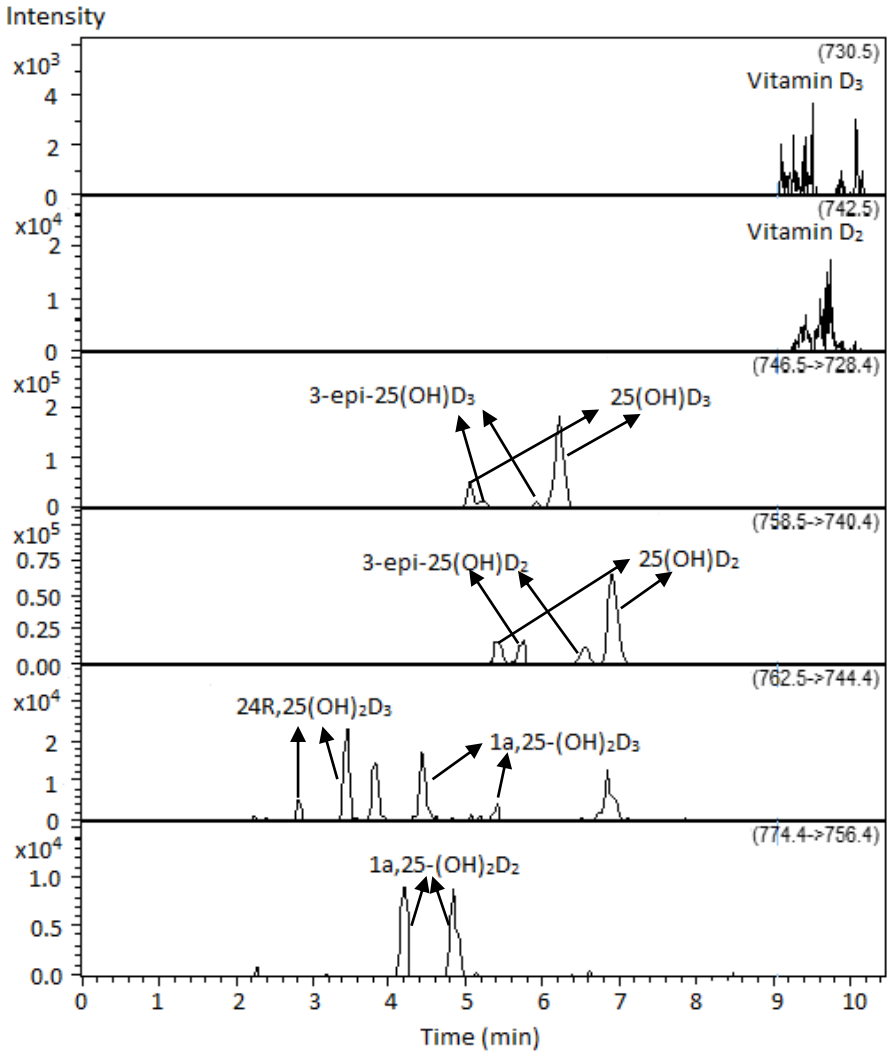


Figure 4-4. Chromatograms of the 4th standard in the HPLC-MS/MS analysis of the 1st run of Soay sheep samples. The concentrations for vitamin D_2, vitamin D_3, 25(OH)D_2, 25(OH)D_3, 1α,25-(OH)$_2D_2$, 1α,25-(OH)$_2D_3$, 3-epi-25(OH)D_2, 3-epi-25(OH)D_3, and 24R,25(OH)$_2D_3$ were 22.34, 23.04, 12.89, 35.39, 8.27, 12.76, 4.30, 4.42 and 8.51 nmol/l, respectively.

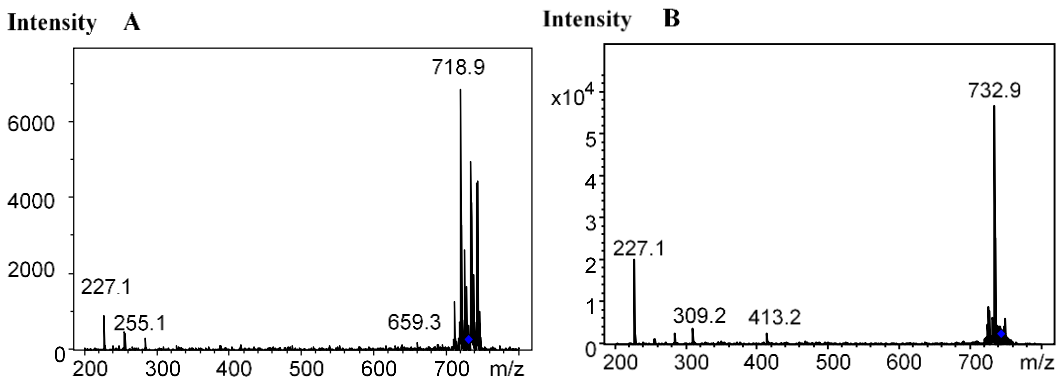


Figure 4-5. Mass spectra of DMEQ-TAD derivative of vitamin D_3 (A) and vitamin D_2 (B) shown in Figure 4-4.

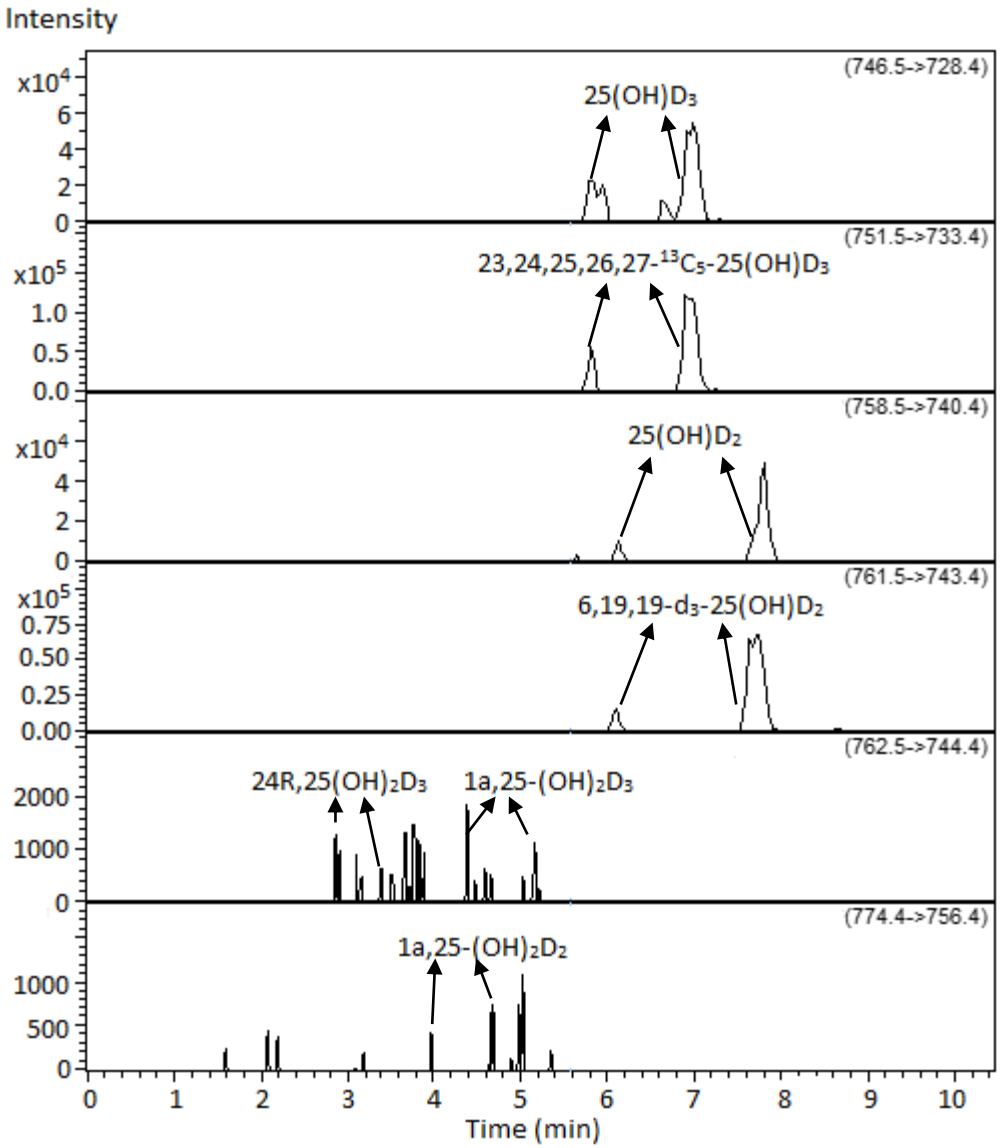


Figure 4-6. Chromatograms of a Soay sheep sample. In the windows for 1α,25-$(OH)_2D_2$, 1α,25-$(OH)_2D_3$ and 24R,25$(OH)_2D_3$, the arrows are used to show the retention time where the relevant peaks should have appeared for the indicated vitamin D metabolite.

4.4.5 HPLC-MS/MS detection of DMEQ-TAD derivatives of 25(OH)D_2 and 25(OH)D_3

The major ions for 25(OH)D_2 and 25(OH)D_3 derivatives were at a mass/charge (m/z) ratio of 758.5 and 746.5, respectively. Under optimized HPLC-MS/MS conditions, each of these derivatives produced two main fragments – a DMEQ-TAD fragment with m/z ratio of 247.0 and A-ring/DMEQ-TAD with m/z ratio of 468.1 (Figure 4-7

and Figure 4-8). Both fragment ions were used in the MRM during tandem mass spectrometry detection. In the final HPLC-MS/MS method, derivatised 25(OH)D_2 and 25(OH)D_3 were eluted at 5.7 & 7.3 min and 5.4 & 6.6 min, respectively (Figure 4-9) during the 10 min analysis. The areas of the major peaks, these being 6S isomers in both cases, eluted at 7.3 min for 25(OH)D_2 and at 6.6 min for 25(OH)D_3, were used for quantitation.

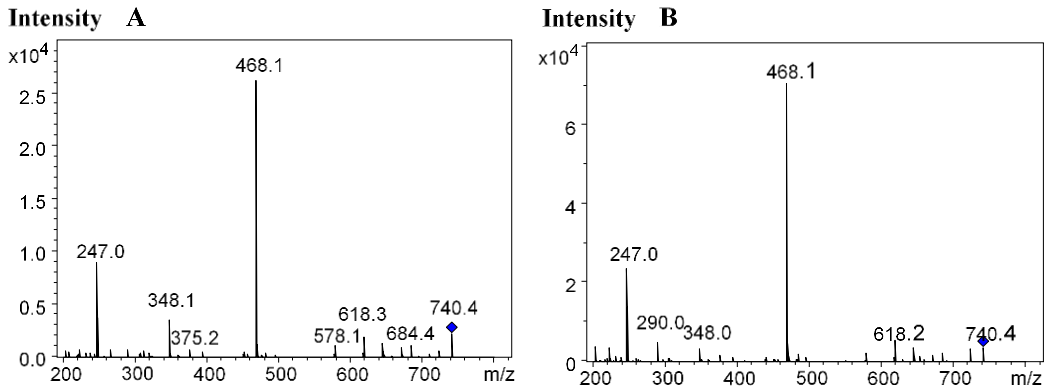


Figure 4-7. Mass spectra of DMEQ-TAD derivative of 25(OH)D_2 shown in Figure 4-9 (A is for the peak at 5.7 min, B is for the peak at 7.3 min), which indicate that fragments with m/z of 247.0 and m/z of 468.1 were the main fragments of the 25(OH)D_2 derivative.

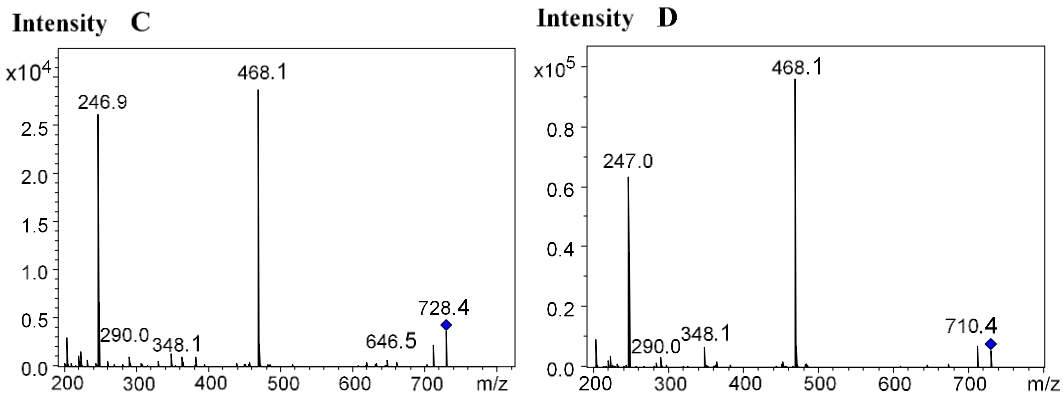


Figure 4-8. Mass spectra of DMEQ-TAD derivative of 25(OH)D_3 shown in Figure 4-9 (C is for the peak at 5.4 min, D is for the peak at 6.6 min), which indicate that fragments with m/z of 247 and m/z of 468.1 were the major fragments of the 25(OH)D_3 derivative.

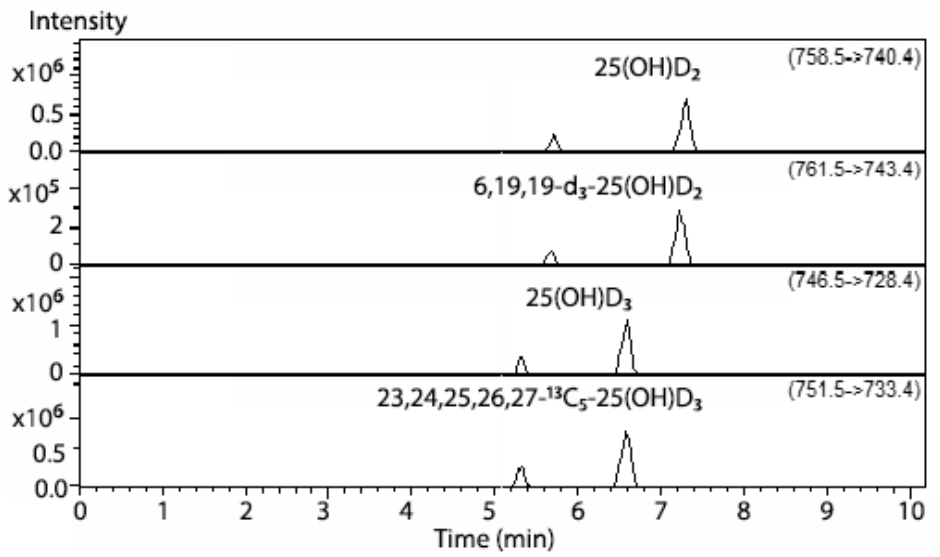


Figure 4-9. Chromatograms of a calibration standard of 25(OH)D_2 (28.85 nmol/l) and 25(OH)D_3 (44.58 nmol/l). MRM transition was at 758.5>468.1 for 25(OH)D_2, and at 746.5>468.1 for 25(OH)D_3.

4.4.6 Method validation

The recovery rate was 63% for 25(OH)D_2 and 54% for 25(OH)D_3. LLOD and LLOQ of 25(OH)D_2 were 3.6 nmol/l and 7.2 nmol/l, respectively; while LLOD and LLOQ of 25(OH)D_3 were identical, both being 5.6 nmol/l. The mean intra-assay coefficients of variation were 7.6% for 25(OH)D_2 and 7.7% for 25(OH)D_3; the mean inter-assay coefficients of variation were 17.4% for 25(OH)D_2 and 15.1% for 25(OH)D_3 (Table 4-5). The average injection carryovers were 0.07% (ranged from undetectable to 0.22% in blank), 0.09% (ranged from undetectable to 0.41% in blank), 0.14% (ranged from undetectable to 0.36% in blank) and 0.32% (ranged from undetectable to 0.85% in blank) for 25(OH)D_2, 25(OH)D_3 and their corresponding ISs, respectively.

The concentrations of 25(OH)D_2 and 25(OH)D_3 of 10 Soay sheep serum samples detected in the current experiment were well associated with their corresponding results reported previously from an accredited lab (see Appendix 4-3 for concentrations of 25(OH)D_2 and 25(OH)D_3 of 10 Soay sheep serum samples). The Pearson correlation coefficients calculated for the two sets of results were 0.97 ($P<0.001$) and 0.86 ($P=0.001$) for 25(OH)D_2 and 25(OH)D_3, respectively.

Table 4-5. Intra assay and inter assay coefficients of variation for 25(OH)D_2 and 25(OH)D_3.

		Intra assay					Inter assay			
		Run					Sheep sample			
		1	2	3	4	5	A	B	C	D
25(OH)D_2	**Mean (nmol/l)**	-	-	-	-	-	23.21	26.11	22.48	32.11
	SD	-	-	-	-	-	3.51	4.04	5.70	4.29
	CV	8.9	7.6	6.5	6.6	8.5	15.1	15.5	25.4	13.4
25(OH)D_3	**Mean (nmol/l)**	-	-	-	-	-	19.35	32.09	22.63	33.79
	SD	-	-	-	-	-	2.15	6.36	0.87	8.63
	CV	6.8	5.6	6.7	8.5	10.8	11.1	19.8	3.9	25.6

4.5 Discussion

This newly derived HPLC-MS/MS method enabled quantification of 25(OH)D_2 and 25(OH)D_3 concentrations in serum samples collected from sheep in North West Scotland. These two vitamin D metabolites are the key vitamin D derivatives that have been commonly measured to indicate vitamin D status, firstly due to their much higher circulating concentrations compared to those of other vitamin D metabolites (Bikle, 2018). Secondly, they have a longer physiological half-life (approximately 14-21 days; Holick, 2009) than other vitamin D metabolites, this being for example just 36 to 48 hours for vitamin D_3 (Avioli et al., 1967) or approximately 4 to 6 hours for 1α,25-$(OH)_2$D (Holick, 2009). Thirdly, concentration of 25(OH)D is less tightly regulated by parathyroid hormone. Consequently, 25(OH)D has been considered a reliable indicator for determination of vitamin D status (Holick, 2009).

Although the current method could not determine the concentrations of the C3-epimers of 25(OH)D, these epimers were separated from their precursors – 25(OH)D_2 and 25(OH)D_3 (Figure 4-3) – which have the same molecular weights and MRM transitions as their respective C3-epimers (Table 4-3). This separation was deemed necessary because a study of serum from human infants (less than 1-year-old) showed that the concentration of 3-epi-25(OH)D can confound the determination of serum 25(OH)D status when using HPLC-MS/MS, and lead to overestimation of actual concentrations (Singh et al., 2006). Later, Strathmann et al. (2012) reported that measuring 25(OH)D_3 concentration without exclusion of the concentration of 3-epi-25(OH)D_3 could misclassify 9% and 3% of human patients

(<1 year and 1 to 94 years, respectively) as vitamin D sufficient. By chromatographically separating 25(OH)D and its C3-epimers, the results obtained using the current method should display the status specifically of 25(OH)D without distortion by its C3-epimers.

Derivatization with particular chemical reagents can increase the molecular weight and thereby help to enhance signal to noise ratio; this reaction can also modify the physical structure of the analyte to improve its chromatographic behaviour, whereby the sensitivity and selectivity of determination of analyte in HPLC-MS/MS analysis can be improved (Higashi & Shimada, 2017). There are many different types of derivatization reagents available, such as Cookson-type reagents (e.g. DMEQ-TAD), which have the powerful dienophile, TAD, that reacts with the conjugated diene group from the analyte to form a stable and permanently charged Diels-Alder derivative. This derivative has a higher proton affinity, and provides a higher response than the intact analyte, during tandem mass spectrometry detection with positive electrospray ionization mode (Higashi & Shimada, 2017). Shimizu et al. (1991) reported that the efficiency of the reaction between vitamin D metabolites and DMEQ-TAD was high, and that the benefit of derivatisation with DMEQ-TAD was due to the fact that the derivatives of vitamin D metabolites were more stable compared to the intact ones, as their light- and oxygen-sensitive conjugated triene group was disguised in the derivatives.

The hydrophobic nature of vitamin D molecules, combined with the lack of ionisable groups in their chemical structure, leads to a low efficiency of ionization of vitamin D metabolites during HPLC-MS/MS assay. However, because vitamin D metabolites possess a conjugated diene group (Higashi & Shimada, 2017), derivatization with Cookson-type reagent can improve the detection of vitamin D metabolites in the mass spectrometer. Higashi et al (2001) and Kaufmann et al (2014) reported that derivatization with DMEQ-TAD could increase, by 10-fold or more, the sensitivity of determination of vitamin D metabolites using HPLC-MS/MS. The method used in the current study involved direct derivatization with DMEQ-TAD, which increases molecular weight by 363 Da (Kaufmann et al., 2014), and improves ionization efficiency compared to that achieved with native metabolites (Figure 4-1). Moreover, derivatization of 25(OH)D_2 and 25(OH)D_3 with DMEQ-TAD generated, in both cases, two isomers (i.e. 6S and 6R), due to the reaction with a *s-cis*-diene moiety

from both α and β sides, and therefore, two peaks were presented for each metabolite on the chromatogram (Higashi and Shimada, 2017). The ratios of 6S to 6R (7:2 and 3:1 for 25(OH)D_2 and 25(OH)D_3, respectively) were either in agreement or similar to the ratio (7:2 for 25(OH)D) reported by Higashi et al (2001). In the current experiment, the major peak for the 6S isomer was used for quantification, consistent with earlier studies (Higashi et al., 2001; Kaufmann et al., 2014). Under the optimized HPLC conditions, the 25(OH)D_2 and 25(OH)D_3 derivatives generated two main fragments with m/z ratios of 247.0 and 468.1 (Figure 4-7 and Figure 4-8). This also was consistent with the report of Kaufmann et al (2014). The LLOD of this method were 3.61 nmol/l for 25(OH)D_2 and 5.57 nmol/l for 25(OH)D_3, which were in the recognised range for the detection limits (3-7.5 nmol/l) when using HPLC-MS/MS for detecting the same vitamin D metabolites (reviewed by Wallace *et al.* 2010). The LLOQ of this HPLC-MS/MS method were 7.22 nmol/l for 25(OH)D_2 and 5.57 nmol/l for 25(OH)D_3, which were below the quantification limits range (10-17.5 nmol/l) for 25(OH)D_2 and 25(OH)D_3 noted by Wallace et al. (2010) for HPLC-MS/MS. However, the LLOQs reported here were higher than those (2ng/ml (equivalent to 4.85 nmol/l) for 25(OH)D_2 and 2 ng/ml (equivalent to 4.99 nmol/l) for 25(OH)D_3) determined by Fritz et al. (2017) using HPLC-MS/MS (an Agilent HPLC system coupled to a Sciex 4000 QTrap). The divergence could be due to the criteria applied for determining LLOQ were different, as the minimum signal to noise ratio for the current method was 10:1, while it was 5:1 in the method reported by Fritz et al. (2017). Also, the different sensitivities of the instruments used might have influenced the LLOQ results being reported.

The recovery rates were 63% for 25(OH)D_2 and 55% for 25(OH)D_3, which were in the range of satisfactory results (55-85%) reported by Ding et al. (2010). The injection carryover was determined in the manner suggested by Fritz et al. (2017), and the results for 25(OH)D_2 and 25(OH)D_3 were in the ranges (<0.1%) that the same authors reported. The intra-assay CV for 25(OH)D_2 (7.6%) and 25(OH)D_3 (7.7%) complied with EMA and FDA guidelines for Bioanalytical Methods (US Food and Drug Administration, 2001; European Medicines Agency, 2012), whereas the respective inter-assay CV (17.4% and 15.1%) did not quite meet the recommended standard (i.e. minimum CV of 15%; (US Food and Drug Administration, 2001; European Medicines Agency, 2012)). These data indicate that the levels of precision between different runs were less satisfactory. This might be explained by variable

performance of both the equipment and the operator, especially since the HPLC-MS/MS had been intensively used for more than 5 years, suggesting that its sensitivity may have declined; moreover, the operator (this author) had had no prior experience of HPLC-MS/MS. Also, variability in reagents and ambient conditions could have been contributors to the CV values recorded. Subjectively, while acknowledging that precision could be expected to be improved with greater experience and new equipment, performance was acceptable under the circumstances.

In terms of considering the accuracy (distinct from precision) of the analyses, it is notable that Pearson tests of association between two sets of vitamin D metabolite analysis results for Soay sheep serum showed that there was very good association between results obtained from application of the current method and the corresponding results reported by an accredited laboratory in Manchester (see Section 4.4.6). Vitamin D metabolites are reported to be quite stable in human plasma and serum, according to previous studies on the stabilities of vitamin D metabolites (Lissner et al., 1981; Antoniucci et al., 2005; Wielders & Wijnberg, 2009; Müller et al., 2016). The Soay sheep serum samples analysed using the current method had been stored at -80°C for approximately 5 years, so there might have been some degradation of vitamin D metabolites in those samples, albeit to our knowledge no research has investigated the stability of vitamin D metabolites after long term-storage. While not in any way definitive, the very good association ($r > 0.95$) between analyses conducted several years apart suggests that the vitamin D metabolites were indeed stably preserved in Soay serum samples since collection in 2012.

HPLC-MS/MS is recommended as a ‘gold standard’ method for determination of vitamin D metabolites in biological samples (Volmer et al., 2015), and the technique can synchronously quantify several vitamin D metabolites due to its high selectivity (Jenkinson et al., 2016). The limitation of the current method is that only two main circulating forms of vitamin D, i.e. $25(OH)D_2$ and $25(OH)D_3$, could be examined in sheep serum samples. If time and budget allowed during the method development, more investigations for comparing different 96-well SPE plates (e.g. C8 sorbent for SPE: Mena-Bravo et al., 2015), or different derivatisation reagents (e.g. Amplifex: Hedman et al., 2014; or 2-nitrosopyridine: Wan et al., 2017), or different HPLC

columns (e.g. Lux Cellulose-3 Chiral column: Jenkinson et al., 2016), could have been undertaken. These modifications might have improved the method performance, allowing more vitamin D metabolites to be quantified, and a better limit of quantification to be achieved. Crucially, however, informative analysis of the key metabolites, 25(OH)D_2 and 25(OH)D_3, has been achieved.

4.6 Conclusion

This experiment developed and evaluated a HPLC-MS/MS method specifically for determination of vitamin D status in sheep serum samples. The limits of detection, limits of quantification, injection carryover and inter assay and intra assay coefficients of variation were assessed, and these parameters were consistent with previous reports and indicated that this HPLC-MS/MS method is a suitable procedure for determining 25(OH)D_2 and 25(OH)D_3 concentrations in sheep serum samples. It was therefore used to examine 25(OH)D_2 and 25(OH)D_3 concentrations in sheep serum samples collected in November 2015 at the SRUC Hill and Mountain Research Centre, the subject of research reported in Chapter 5.

Chapter 5: Investigation of relationship between vitamin D status and reproductive fitness in Scottish hill sheep

5.1 Introduction

The main interest of this PhD study was to investigate whether farming Lleyn ewes, a lowland/upland prolific sheep breed, in a Scottish hill environment can improve productivity of hill sheep farming enterprises. In order to do this, the performance of Lleyn ewes farmed on a Scottish hill farm was compared with the performance of a typical hill breed, BF flockmates from two different genetic lines (i.e. UBF and IBF), as described in the previous chapters. Several possible causes that might lead to performance difference among the three genetic lines were also investigated. One further potential cause could be the ewes' vitamin D status, as several studies in humans reported that maternal vitamin D deficiency was associated with an increased risk of pregnancy loss (Mumford et al., 2018), or newborn babies being small for gestational age (Gernand et al., 2013; Chen et al., 2017) and having low birth weight (Wang et al., 2018). A study of Soay sheep, in a feral population on a remote Scottish island, showed that ewe 25(OH)D (the sum of $25(OH)D_2$ and $25(OH)D_3$ as described in Chapter 1) serum concentrations were positively associated with the number of lambs reared to one year old (Handel et al., 2016): this is an important ewe reproductive performance criterion.

Exposure to sunlight that contains sufficient ultraviolet B radiation is a key factor influencing endogenous photobiosynthesis of vitamin D (Scientific Advisory Committee on Nutrition, 2016). The study farm for this PhD project is located in a high latitude region (56ºN, 4ºW), where the amount of ultraviolet B radiation available and necessary for facilitate vitamin D photobiosynthesis (wavelength 290-315 nm; Holick, 2007) is low between October and March (Scientific Advisory Committee on Nutrition, 2016). Low quality and quantity of ultraviolet B radiation during that time of the year has been shown to result in no previtamin D_3 being synthesized from 7-Dehydrocholesterol in human skin, even with exposure to sunlight (Webb et al., 1988). This could also affect the vitamin D status of sheep farmed in those regions. Additionally, the current study flock comprised two different

sheep breeds with different skin pigmentations (unpigmented in the Lleyn breed and black and unpigmented in the BF breed), that could lead to different efficiencies of vitamin D photobiosynthesis in their skin (Holick, 1981; Mearns et al., 2008). To the best of this author's knowledge, the effects of vitamin D status on reproductive performance of domesticated sheep have not been published; to help redress this, the current study flock provided a unique opportunity to research this topic.

This chapter compares serum 25(OH)D_2 and 25(OH)D_3, and consequently total 25(OH)D concentrations among the three ewe genetic lines using the HPLC-MS/MS method demonstrated in Chapter 4. The associations between vitamin D status (25(OH)D_2, 25(OH)D_3 and 25(OH)D concentrations) and ewe reproductive traits, i.e. number of lambs born and weaned, litter weight (including singletons and twins) at birth, marking and weaning were investigated. In the statistical analyses, the number of lambs weaned in the last breeding cycle was considered in the models for analysing vitamin D status (25(OH)D_3 and 25(OH)D concentrations) and ewe breeding outcomes to examine, if there were any effects of ewe previous reproductive performance on these response variates investigated. Moreover, the lambing percentages reported in this publication were based on the ewes selected for determination of vitamin D concentrations, not the whole study flock. A higher percentage of barren ewes' serum samples (compared with those for single- and twin-bearing ewes) were submitted for vitamin D analyses, which avoided under-representation in that cohort within this assay, if barrenness was linked with ewe vitamin D status.

The following publication was written by the author of this thesis with contributions and minor revisions from co-authors and reviewers. It is published in Scientific Reports (Zhou, P., McEvoy, T.G., Gill, A.C., Lambe, N.R., Morgan-Davies, C.R., Hurst, E., Sargison, N.D. and Mellanby, R.J. (2019) Investigation of relationship between vitamin D status and reproductive fitness in Scottish hill sheep. *Scientific Reports*, 9:1162 (DOI https://doi.org/10.1038/s41598-018-37843-6)). In this study, all work was performed by this author except individual contributions as acknowledged in the published paper and by co-authorship.

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Investigation of relationship between vitamin D status and reproductive fitness in Scottish hill sheep

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Ping Zhou[1,2], Thomas G. McEvoy[2], Andrew C. Gill[1,3], Nicola R. Lambe[2], Claire R. Morgan-Davies[2], Emma Hurst[1], Neil D. Sargison[1] & Richard J. Mellanby[1]

There is a growing interest in the influence of vitamin D on ovine non-skeletal health. The aim of this study was to explore the relationship between pre-mating vitamin D status, as assessed by serum concentrations of 25-Hydroxyvitamin D [25(OH)D; comprising D_2 and D_3] and subsequent reproductive performance of genetically unimproved Scottish Blackface (UBF), genetically improved Scottish Blackface (IBF) and Lleyn ewes kept under Scottish hill conditions. 25-Hydroxyvitamin D_2 (25(OH)D_2) and 25-Hydroxyvitamin D_3 (25(OH)D_3) concentrations were determined in serum samples harvested in November from ewes grazed outdoors. There were no significant differences in 25(OH)D_2 concentrations amongst the 3 genotypes. Lleyn ewes had significantly higher 25(OH)D_3 and 25(OH)D concentrations than both Scottish Blackface ewe genotypes, whereas these vitamin D parameters did not differ significantly between the UBF and IBF ewes. Concentrations of 25(OH)D_3 and 25(OH)D were positively associated with subsequent birth weights of singleton and of twin lamb litters. No significant associations between vitamin D status and number of lambs born or weaned per ewe were found. This study demonstrates that concentrations of cutaneously-derived 25(OH)D_3, but not of orally consumed 25(OH)D_2, differed between breeds. The positive association between ewe vitamin D status and offspring birth weight highlights the need for further investigations.

The two forms of vitamin D, namely D_2 and D_3, can be obtained from diet or during sunlight exposure. Vitamin D_2 and its precursor can be found in fungal resources, such as wild mushroom (e.g. *Chantarellus tubaeformis*)[1], whereas the vitamin D_3 content of certain animal species, such as wild salmon, is high[2]. During exposure to ultraviolet B radiation (wavelength, 290–315 nm), 7-Dehydrocholesterol in the skin is converted to previtamin D_3 and then vitamin D_3. After vitamin D enters the systemic circulation, it is hydroxylated in the liver to the main circulating form, 25-Hydroxyvitamin D (25(OH)D). This metabolite is further hydroxylated in the kidneys to the biologically active form, 1,25-Dihydroxyvitamin D (1α,25-$(OH)_2$D)[3]. Compared to 1α,25-$(OH)_2$D, the concentration of 25(OH)D is a more reliable indicator for determining vitamin D status, due to its longer half-life, a higher serum concentration and the fact that it is less tightly regulated by parathyroid hormone[4,5].

Endogenous photobiosynthesis of vitamin D depends on exposure to sunlight which contains sufficient ultraviolet B radiation[6]. The quality and the quantity of ultraviolet B radiation are affected by latitude and season, as when the sun is low in the sky, more ultraviolet B radiation is scattered and absorbed when it travels through the ozone layer, compared to when the sun is directly overhead[7]. In regions where the latitudes are above 39°N, such as the UK (from 49 to 60°N), the low level of ultraviolet B radiation results in no previtamin D_3 being synthesized from 7-Dehydrocholesterol in human skin during exposure to sunlight, from October to March[6–8]. This could also lead to low vitamin D status in sheep farmed in such high latitude locations, although literature quantifying optimal ultraviolet radiation levels for sheep is difficult to find.

[1]Royal (Dick) School of Veterinary Studies and The Roslin Institute, The University of Edinburgh, Easter Bush Veterinary Centre, Roslin, Midlothian, EH25 9RG, UK. [2]SRUC (Scotland's Rural College), King's Buildings, West Mains Road, Edinburgh, EH9 3JG, UK. [3]School of Chemistry, Joseph Banks Laboratories, University of Lincoln, Green Lane, Lincoln, LN6 7DL, UK. Correspondence and requests for materials should be addressed to R.J.M. (email: Richard.Mellanby@ed.ac.uk)

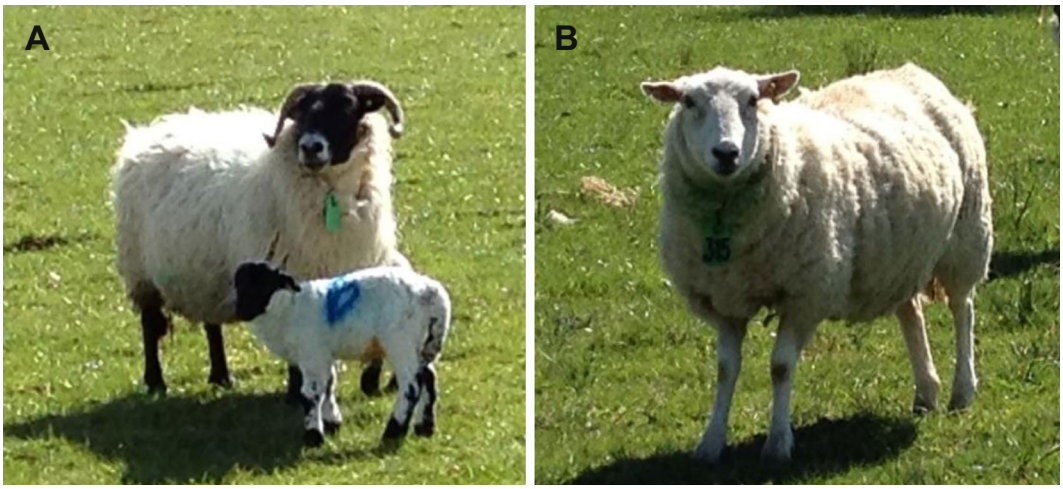

Figure 1. Photographs of Scottish Blackface (**A**) and Lleyn (**B**) ewes at SRUC Hill and Mountain Research Centre (photographs taken by Ping Zhou).

The principal function of vitamin D is the maintenance of skeletal health through regulating the processes of intestinal absorption and renal excretion of calcium and phosphorus, bone formation and mineral mobilization[3]. However, in the last 2 decades, numerous studies have reported that vitamin D deficiency is associated with many non-skeletal health problems, such as autoimmune diseases, hypertension and cancer[9,10]. The presence of vitamin D receptors in the reproductive tract of women[11] and females of other species, such as sheep[12], goat[13], mouse[14,15] and rat[16], and in ovine male reproductive tracts[17,18] indicates that vitamin D may influence reproductive performance. Several human studies have found that vitamin D deficiency before and during pregnancy is associated with reduced reproductive success and increased risk of the newborn being small for gestational age, lighter in weight at birth, or having reduced head circumference[19–22]. A recent ovine study suggests a role for vitamin D_3 in spermatogenesis[18].

The Scottish Blackface sheep (Fig. 1A) is a hardy hill breed with a white fleece, and mostly black skin and hair on the face and legs, whereas the Lleyn sheep (Fig. 1B) is a lowland/upland prolific breed with white fleece, non-pigmented skin and white hair on its face and legs. The Scottish Blackface is native to Scotland and is the most common breed found in the extensive hill farms of the west coast, where the annual number of sunshine hours is low, during autumn and winter, the monthly average daylight hours ranging from 10 hours 20 minutes in October to 7 hours in December[23], whereas Lleyn sheep are native to Wales. We investigated the hypothesis that there is a breed-dependent effect on vitamin D status of sheep in North West Scotland (56°N) and that there is a positive relationship between ewe reproductive performance and vitamin D status. To investigate this hypothesis, serum 25(OH)D_2 and 25(OH)D_3 concentration of three populations of ewes were determined using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). The relationship between vitamin D status of contrasting genotypes, all in the same flock, and breeding outcomes was investigated.

Materials and Methods

Study flock. The experiment was conducted at Scotland's Rural College (SRUC) Hill and Mountain Research Centre, Crianlarich, Scotland (56°N, 4°W; elevation: 170 to 1025 m; mean annual rainfall: 3,000 mm)[24]. The study flock comprised approximately 200 genetically unimproved Scottish Blackface (UBF) ewes, 200 genetically improved Scottish Blackface (IBF) ewes and 200 Lleyn ewes. The UBF ewes had been selected in each generation to remain close to the average genetic merit in the flock before selection commenced in 1998, while IBF ewes were a lineage progressively selected for superior genetic merit in both ewe and lamb traits[25]. The Lleyn ewes had been selected for high genetic merit since 2010 using the Carcass+ Index (which aims to identify sheep with superior breeding potential for maternal ability, lamb growth and carcase quality) as part of the Signet Sheepbreeder performance recording service (www.signetfbc.co.uk/sheepbreeder/).

Prior to blood sampling (in autumn 2015), the research flock was drawn equally from two 'pre-study' management system groups, one group having been managed conventionally (CON), and the other subjected to a Precision Livestock Farming (PLF) management protocol. Each system had different criteria for winter feeding, worming and culling[24,26]. Ewes in each system had shared the same pastures. From mating 2015, the flock was subject to two management systems, both of which used the PLF management approach previously developed[24,26]. Ewes were assigned to either a predominantly 'Hill Grazing' or 'Park Grazing' management system (Supplementary Fig. S1), with each system having different strategies for using grazing resources and feed supplements.

Ewes were mated in single-sire mating groups. There were 4 single-sire mating groups per genetic line. The mating group size in 2015 ranged from 44 to 50 ewes in each single-sire group. From late-November, ewes were joined with rams selected from their own genotype category for two reproductive cycles of 17 days. Ewes were ultrasound pregnancy-scanned to determine pregnancy status and foetal numbers in mid-February. Two supplementary feeding levels (Supplementary Table S1), either "standard" or more generous "corrective", were provided in two phases, to help meet ewe intake requirements[27]. The first feeding phase was from early January

(6^{th}/7^{th} Jan 2016) to scanning (22^{nd}/23^{rd} Feb 2016), while the second feeding phase was from scanning to lambing (Supplementary Fig. S1), which began on 15^{th} April. All sheep, depending on weight, body condition score (CS) and pregnancy diagnosis, were assigned to one or other in each phase.

Data were collected for all ewes in the flock and included production (e.g. ewe weight and CS), and reproduction (e.g. pregnancy and lambing) records, as well as individual and group health treatments. Ewes were gathered at pre-mating, mid-pregnancy scanning and pre-lambing. At these handling events, ewes were weighed, and condition scored using a 5 point scoring system[28]. Lambs were tagged within 24 hours after birth, and data were collected on birth weight, sex and litter size. Lamb weights were also recorded at around 8 weeks of age (marking, end of June) and at weaning (mid-August).

All experiments had local ethical approval and were conducted in accordance with UK legislation. The experimental protocols involving animals were approved by the SRUC Animal Welfare and Ethical Review Body.

Sample collection. In mid-November 2015, during pre-mating handling, blood samples were taken via jugular venepuncture into 6 ml silicone coated red-top blood collection tubes (BD, Plymouth, UK). Ewes were between 1.5 and 6.5 years-old at the time of sampling (Supplementary Table S2). Samples were kept on ice during transportation from farm to the laboratory in Edinburgh for processing. The tubes were centrifuged at 3500 rpm at 4 °C for 10 minutes. Serum was then removed into 2 ml screw capped micro-tubes. The 0.5 ml serum aliquots were stored at −20 °C overnight, then stored at −80 °C until analysis.

Determination of 25(OH)D_2 and 25(OH)D_3 concentrations. Blood samples of 88 ewes per genotype were analysed to determine pre-mating ewes' 25(OH)D_2 and 25(OH)D_3 serum concentrations.

Calibration standards. Eight calibration standards were freshly prepared, by adding 20 µl of 25(OH)D_2 stock solution (5 µg/ml in ethanol; Sigma-Aldrich, UK) and 30 µl of 25(OH)D_3 stock solution (5 µg/ml in ethanol; Sigma-Aldrich, UK) into 1 ml artificial serum [50 mg bovine serum albumin (Sigma-Aldrich, UK) were dissolved in 1 ml of phosphate buffered saline (prepared in house)], then 1 in 2 serial dilution with artificial serum. The concentrations of calibration standards were 230.8, 115.4, 57.7, 28.9, 14.4, 7.2, 3.6 and 1.8 nmol/l for 25(OH)D_2; and 356.6, 178.3, 89.2, 44.6, 22.3, 11.2, 5.6 and 2.8 nmol/l for 25(OH)D_3. These calibration standards were used to generate standard curves for quantification of the concentration of 25(OH)D_2 and 25(OH)D_3 in sheep serum by HPLC-MS/MS analysis.

Sample preparation. After serum samples (0.5 ml) were thawed at room temperature, 100 µl of each sample, or calibration standard, was spiked in a 1.5 ml microtube with 2 µl of 6,19,19-d_3-25(OH)D_2 (1.78 µmol/l; Sigma-Aldrich, UK) and 2 µl of 23,24,25,26,27-$^{13}C_5$-25(OH)D_3 (2.47 µmol/l; Sigma-Aldrich, UK), as internal standards. After adding 20 µl of 1 M NaOH, each serum sample or calibration standard was then protein precipitated by the addition of 200 µl of acetonitrile[29]. The supernatant of the serum sample or the calibration standard was purified by solid phase extraction using a Discovery DSC-18 SPE-96 Plate (bed weight: 25 mg/well; Sigma-Aldrich, UK). Briefly, the plate was activated with 3 ml of ethyl acetate, 3 ml of methanol and 3 ml of distilled water. After addition of a mixture of supernatant (approximately 300 µl) from protein precipitation and 1 ml 0.4 M K_2HPO_4, the plate was washed with 3 ml of distilled water and 2 ml of 40% methanol sequentially and eluted with 1.5 ml of acetonitrile[29]. After evaporating to dryness, samples were derivatized by 2 additions of 25 µl of 0.1 mg/ml DMEQ-TAD (4-[2-(3,4-Dihydro-6,7-dimethoxy-4-methyl-3-oxo-2-quinoxalinyl)ethyl]−3*H*-1,2,4-triazole-3,5(4 *H*)-dione; Abcam, UK) in ethyl acetate[30]. After evaporation to dryness, derivatized extracts were reconstituted in 25 µl of 60:40 (vol:vol) methanol and 0.1% formic acid:water for HPLC-MS/MS analysis.

HPLC-MS/MS analysis. The HPLC-MS/MS analyses were conducted using an UltiMate 3000 HPLC system interfaced to an amaZon ETD tandem mass spectrometer (Bruker Daltonics, Bremen, Germany). Chromatographic separations were achieved using an ACE UltraCore 2.5 SuperC18 column (75 × 2.1 mm, 2.5 µm; Advanced Chromatography Technologies, UK), maintained at 40 °C. Gradient elution was performed (Supplementary Table S3), with the mobile phase consisting of 10 mM ammonium formate (Fisher Scientific) with 0.15% formic acid (buffer A) and methanol with 0.1% formic acid (buffer B). The elution was detected using multiple reaction monitoring with positive electrospray ionisation (Supplementary Table S4 and Supplementary Fig. S2). The total runtime was 12 min per sample.

HPLC-MS/MS analyses of sheep serum samples were conducted on different dates on batches of samples comprised of 8 calibration standards and 24 sheep serum samples. Quantitation was carried out using QuantAnalysis 2.0 software (Bruker Daltonics, Bremen, Germany). The standard curve was generated based on the ratio of the peak area of the standard to that of the corresponding internal standard (Supplementary Table S5). Method performance, i.e. injection carryover, sample preparation recovery, intra-assay coefficient of variation and inter-assay coefficient of variation were determined (Supplementary Table S6).

Statistical analysis. Statistical analyses were conducted using GenStat 16 statistical package (VSN International Ltd. UK). Generalized Linear Models (GLM) were used to investigate the effects of multiple independent variables. Stepwise regression was used to determine the fixed effects and relevant covariates to include in the final model for each response variate. These models were then applied in Linear Mixed Models (LMM), alongside appropriate random effects, to investigate: i) differences in vitamin D concentrations between breeds and among genotypes; and ii) any associations between vitamin D status and ewe breeding outcomes, as well as ewe litter weight at birth, marking and weaning (see Supplementary Table S7 for summary). Statistical significance was defined as $P < 0.05$. When model terms were significant, pairwise Student's t-tests were performed to test for significant differences between different levels of each factor.

Genotype	Sample size	25(OH)D_2 (nmol/l)	25(OH)D_3 (nmol/l)	25(OH)D (nmol/l)
UBF	83	18.6 ± 0.7	19.4 ± 0.9	38.0 ± 1.3
IBF	88	20.0 ± 0.7	19.6 ± 0.9	39.6 ± 1.4
Lleyn	88	20.0 ± 0.6	24.3 ± 1.1	44.3 ± 1.4
Average	—	19.5 ± 0.4	21.1 ± 0.6	40.7 ± 0.8

Table 1. The unadjusted concentrations (mean ± standard error of mean) of 25(OH)D_2, 25(OH)D_3 and 25(OH)D for the 3 genotypes of ewes.

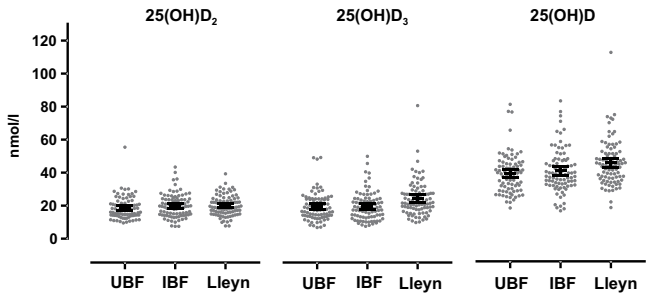

Figure 2. The pre-mating concentration of 25(OH)D_2, 25(OH)D_3 and 25(OH)D of individual ewe samples for the 3 genotypes. Short black lines show the mean with 95% confidence interval.

To investigate differences in vitamin D concentrations [25(OH)D_2, 25(OH)D_3 and total 25(OH)D (addition of 25(OH)D_2 and 25(OH)D_3)] between breeds and amongst genotypes, the ewe breed/genotype, ewe age, pre-study system, ewe pre-mating weight, ewe pre-mating CS, sire of the ewe, number of lambs weaned and weaned litter weight in the last breeding cycle were the factors considered in the maximal GLM models.

In the GLM models used to examine ewe breeding outcomes, the number of lambs born in 2016 and the number of lambs weaned that year, were tested, in turn, as response variates, whilst 25(OH)D_2/25(OH)D_3/25(OH)D concentration, ewe genotype, ewe age, management system (Hill grazing or Park grazing), pre-study system (CON or PLF), ewe pre-mating weight, ewe pre-mating CS, number of lambs weaned in the last breeding cycle (i.e. in 2015) and first winter feeding level were fitted in the maximal models.

When analysing the association of vitamin D status with litter weight at birth, marking and weaning, 25(OH)D_2/25(OH)D_3/25(OH)D concentration, ewe genotype, ewe age, management system, pre-study system, first winter feeding level, ewe pre-mating weight, ewe pre-mating CS and ram group were the factors considered in the maximal models in the GLM analyses. These analyses were conducted separately for single- and twin-bearing ewes.

The final fixed models, selected by stepwise regression, and random effects, fitted for each response variate, are shown in Supplementary Table S7. The random effects were either 'batch' for testing vitamin D status between breeds and amongst genotypes, or 'batch and ram group' for the rest of the LMMs. Batch represented HPLC-MS/MS analysis date. Ram group identified the relevant single sire mating group.

Results

Pre-mating (November 2015) vitamin D concentrations. Analysis of two hundred and sixty-four sheep serum samples for pre-mating ewes' 25(OH)D_2 and 25(OH)D_3 concentrations indicated that the latter differed between breeds. The unadjusted average serum concentrations of 25(OH)D_2, 25(OH)D_3 and total 25(OH)D for the UBF, IBF and Lleyn ewes are shown in Table 1.

The results for five UBF ewes were excluded from the data reported here, as either their measured serum 25(OH)D_3 concentration, or both 25(OH)D_2 and 25(OH)D_3 concentrations were beneath the lower quantification limit (7.2 and 5.6 nmol/l for 25(OH)D_2 and 25(OH)D_3, respectively) of the assay as applied in the current experiment.

Eighteen ewes (6.9%) had 25(OH)D concentrations <25 nmol/l, 238 ewes (91.9%) had concentrations between 25 and 75 nmol/l, and 3 ewes (1.2%) had concentrations >75 nmol/l (Fig. 2).

Serum 25(OH)D_2 concentrations did not differ among either breed ($P = 0.188$) or genotype ($P = 0.263$) after adjusting for age and ewe pre-mating weight (Fig. 3). Serum concentrations of 25(OH)D_2 also were not significantly associated with ewe pre-mating weight or age ($P > 0.05$).

When breed, ewe pre-mating weight, number of lambs weaned and weaned litter weight in the last breeding cycle were accounted for in the LMM, there was a significant difference in 25(OH)D_3 concentration between the Scottish Blackface and Lleyn ewes ($P < 0.001$; Fig. 3A). When genotype ($n = 3$) was fitted, rather than just breed, the LMM on 25(OH)D_3 concentration among the genotypes showed that Lleyn ewes had significantly higher levels of 25(OH)D_3 than UBF and IBF ewes ($P < 0.01$ and $P < 0.001$, respectively; Fig. 3B), whereas the concentration of this vitamin D metabolite did not differ significantly between UBF and IBF ewes ($P > 0.05$; Fig. 3B). Ewe

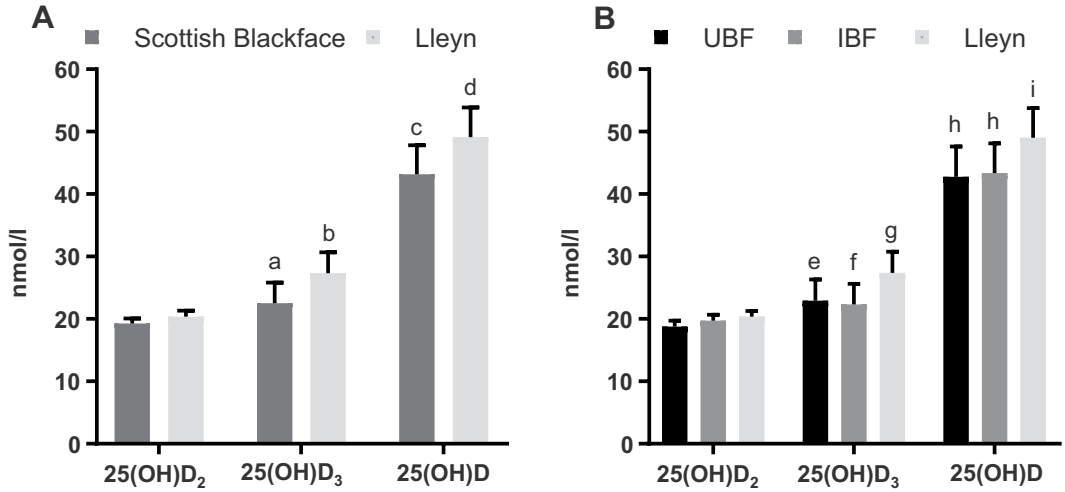

Figure 3. The predicted means ± standard error of 25(OH)D_2, 25(OH)D_3 and total 25(OH)D concentrations (**A**) between breeds and (**B**) among the 3 genotypes. Significant differences denoted as follows: $^{ab}P < 0.001$; $^{cd}P < 0.001$; $^{eg}P < 0.01$; $^{fg}P < 0.001$; $^{hi}P < 0.01$.

		Number of lambs born			Number of lambs weaned		
		0	1	2	0	1	2
25(OH)D_2 (nmol/l)	UBF	18.4 ± 1.5 (17)	18.2 ± 1.3 (39)	19.3 ± 1.1 (27)	18.9 ± 1.3 (20)	18.4 ± 1.2 (43)	18.8 ± 1.2 (20)
	IBF	18.9 ± 1.4 (18)	19.1 ± 1.2 (34)	21.4 ± 1.2 (36)	18.6 ± 1.3 (24)	19.3 ± 1.1 (36)	22.0 ± 1.4 (28)
	Lleyn	19.7 ± 1.4 (19)	19.6 ± 1.0 (33)	20.5 ± 1.1 (36)	19.8 ± 1.2 (24)	19.7 ± 1.1 (31)	20.4 ± 1.2 (33)
25(OH)D_3 (nmol/l)	UBF	22.5 ± 2.4 (17)	17.8 ± 0.9 (39)	19.7 ± 2.0 (27)	22.7 ± 2.0 (20)	17.6 ± 0.8 (43)	19.8 ± 2.6 (20)
	IBF	19.4 ± 2.3 (18)	18.3 ± 1.0 (34)	20.9 ± 1.6 (36)	19.7 ± 1.7 (24)	18.6 ± 1.1 (36)	20.8 ± 1.9 (28)
	Lleyn	24.6 ± 2.2 (19)	24.7 ± 1.8 (33)	23.9 ± 1.9 (36)	24.7 ± 2.0 (24)	24.4 ± 1.8 (31)	24.0 ± 2.1 (33)
25(OH)D (nmol/l)	UBF	41.0 ± 3.0 (17)	36.0 ± 1.7 (39)	38.9 ± 2.5 (27)	41.6 ± 2.6 (20)	36.0 ± 1.6 (43)	38.6 ± 3.2 (20)
	IBF	38.3 ± 3.0 (18)	37.3 ± 1.7 (34)	42.4 ± 2.5 (36)	38.4 ± 2.4 (24)	37.9 ± 1.7 (36)	42.8 ± 3.0 (28)
	Lleyn	44.3 ± 2.9 (19)	44.3 ± 2.2 (33)	44.3 ± 2.5 (36)	44.6 ± 2.4 (24)	44.1 ± 2.4 (31)	44.4 ± 2.7 (33)

Table 2. The unadjusted pre-mating serum concentrations (nmol/l) of 25(OH)D_2, 25(OH)D_3 and 25(OH)D [mean ± standard error of the mean (sample size)] in ewes categorised on the basis of number of lambs born and number of lambs weaned in 2016 for the 3 genotypes.

pre-mating weight was positively associated with 25(OH)D_3 concentration ($P = 0.014$ and $P = 0.013$, respectively, when either breed or genotype was fitted in the model).

Similarly, Lleyn ewes had a significantly higher total 25(OH)D concentration than UBF and IBF ewes ($P < 0.01$ and $P < 0.01$, respectively; Fig. 3B), whereas there was no significant difference in total 25(OH)D concentration between UBF and IBF ewes ($P > 0.05$). Ewe pre-mating weights were positively associated with 25(OH)D concentrations in the models with breed ($P = 0.015$) and genotype ($P = 0.017$) fitted, respectively.

Pre-mating vitamin D status and subsequent breeding outcome. The unadjusted pre-mating 25(OH)D_2, 25(OH)D_3 and 25(OH)D concentrations in UBF, IBF and Lleyn ewes grouped according to subsequent litter sizes at birth and at weaning, are presented in Table 2 and Fig. 4. When ewe genotype, age, pre-study system, management system, ewe pre-mating weight, the number of lambs weaned in the last breeding cycle and first winter feeding level were included in the three LMM models, there were no significant differences in serum 25(OH)D_2 ($P = 0.06$), 25(OH)D_3 ($P = 0.57$) and 25(OH)D ($P = 0.534$) concentrations amongst ewes that, in 2016, were barren or had singles or had twins.

Pre-mating 25(OH)D_2 ($P = 0.130$) concentration was not associated with number of lambs weaned, when this trait was adjusted for genotype, age, management system, pre-study system, ewe pre-mating weight, ewe pre-mating CS, number of lambs weaned in the last breeding cycle and first winter feeding level. In addition, the LMM models with 25(OH)D_3 and 25(OH)D concentrations, fitted with genotype, management system, pre-study system, ewe pre-mating weight, ewe pre-mating CS, number of lambs weaned in the last breeding cycle and first winter feeding level, showed that 25(OH)D_3 ($P = 0.287$) and 25(OH)D ($P = 0.978$) status at pre-mating was not a significant determinant of the number of lambs weaned in August 2016.

Pre-mating vitamin D status and subsequent ewe litter weights at birth, marking and weaning. Among the 259 ewes in this study, 54 (21%) did not produce any lambs; the remainder produced one or two

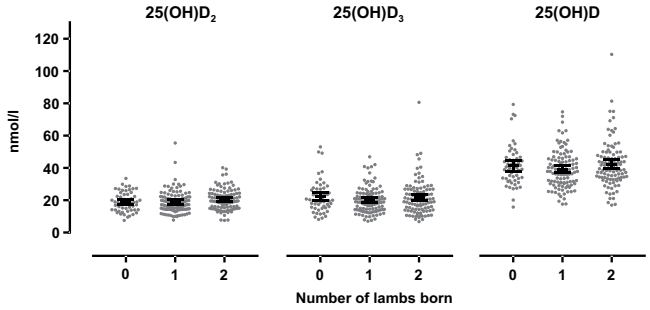

Figure 4. Pre-mating 25(OH)D_2, 25(OH)D_3 and 25(OH)D concentrations recorded for ewes (all 3 genotypes) that subsequently (in 2016) gave birth to 0, 1 or 2 lambs. Short black lines show the means and 95% confidence intervals.

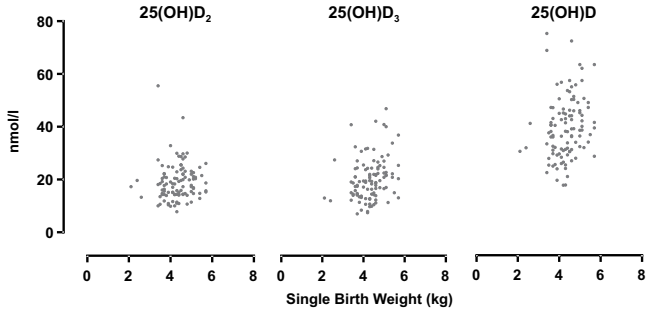

Figure 5. Unadjusted pre-mating 25(OH)D_2, 25(OH)D_3 and 25(OH)D concentrations and subsequent lamb birth weights (kg) of single-bearing ewes (3 genotypes combined).

lambs. The overall lambing percentage was 117%, with 112% for UBF ewes, 120% for IBF ewes and 119% for Lleyn ewes.

Ewes that produced singleton lambs. One hundred and six ewes gave birth to singletons. The associations between the pre-mating vitamin D status of ewes and their subsequent singletons' weights at birth, marking and weaning were investigated, based on all those ewes, including 13 ewes (12%) that lost their lambs between birth and weaning.

There was no relationship between serum 25(OH)D_2 concentration and the birth weight of singletons ($P = 0.355$; Fig. 5), after accounting for the effects of genotype, management system, ewe pre-mating weight and first winter feeding level, whereas 25(OH)D_3 and total 25(OH)D concentrations were positively associated with lamb birth weight of singletons ($P = 0.008$ and $P = 0.017$, respectively; Fig. 5), when genotype, management system and first winter feeding level were accounted for in the LMM models.

For the singleton marking weights, when genotype, management system, first winter feeding level and ewe pre-mating weight were included in the LMM model, none of the 25(OH)D_2 ($P = 0.246$), 25(OH)D_3 ($P = 0.408$), or 25(OH)D ($P = 0.196$) concentrations proved to be a significant factor. When genotype, first winter feeding level and ewe pre-mating weight were considered in the LMM models, 25(OH)D_2 ($P = 0.542$), 25(OH)D_3 ($P = 0.722$) and 25(OH)D ($P = 0.535$) status had no effect on lamb weaning weight.

Ewes that produced twin lambs. Ninety-nine ewes gave birth to twins. The associations between the pre-mating vitamin D status of ewes and their subsequent twin litter weights at birth, marking and weaning were investigated, based on all 99 ewes, including 1 ewe (1%) having no lamb weaned and 17 (17%) ewes having one lamb weaned.

When genotype, age, pre-study system, ewe pre-mating weight and ewe pre-mating CS were considered in the LMM model, the serum 25(OH)D_2 concentration was not a significant determinant of litter birth weight ($P = 0.128$; Fig. 6). Ewe pre-mating serum 25(OH)D_3 and 25(OH)D concentrations were positively associated with litter birth weight of twins ($P < 0.001$ and $P < 0.001$, respectively; Fig. 6), when genotype, management system, ewe pre-mating weight and ewe pre-mating CS were fitted in the LMM models.

At marking, when genotype, management system, first winter feeding level, ewe pre-mating weight and ewe pre-mating CS were included in the LMM, none of 25(OH)D_2 ($P = 0.540$), 25(OH)D_3 ($P = 0.265$), and 25(OH)D ($P = 0.267$) ewe pre-mating serum concentrations showed a significant association with the litter marking weight of the ewes' twin offspring. When genotype, management system, first winter feeding level, ewe pre-mating weight and ewe pre-mating CS were adjusted for in the LMM analyses, none of 25(OH)D_2 ($P = 0.418$), 25(OH)D_3 ($P = 0.355$), and 25(OH)D ($P = 0.297$) had a significant association with the ewes' twin litter weaning weights.

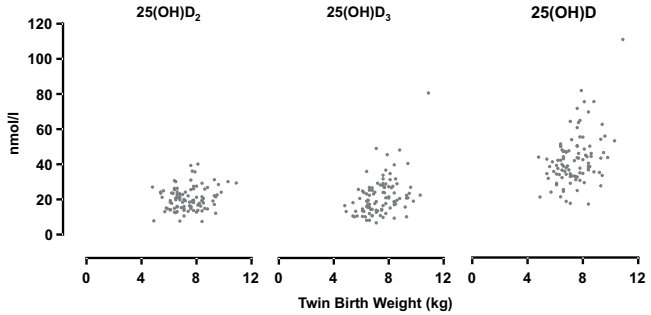

Figure 6. Unadjusted pre-mating 25(OH)D_2, 25(OH)D_3 and 25(OH)D concentrations and subsequent litter birth weights (kg) of twin-bearing ewes (3 genotypes combined).

Discussion

This study has identified a clear relationship between genotype and vitamin D_3, but not D_2, status in a commercially operated sheep farm. Sheep obtain vitamin D_2 from their diet[8]. In this study, the sheep shared the same pastures, and thus the fact that serum 25(OH)D_2 concentration did not differ significantly amongst the 3 genotypes indicates that genotype was not a determinant of vitamin D_2 status within the flock. The concentrations of 25(OH)D_2 recorded for the UBF, IBF and Lleyn ewes (means: 18.6 to 20.0 nmol/l; Table 1) were similar to those of adult ewes (1–6 years old) reported by Handel *et al.*[31], but lower than results reported by Kohler *et al.*[32] from Switzerland (means: 36.7 and 26.0 nmol/l for East Friesian lactating milk sheep kept at altitude of 2,000–2,600 and 400 m for 12 weeks during summer, respectively). Differences could be due to the different blood sampling seasons and contrasting geographical regions with different vegetation in the pasture, which influence herbage vitamin D_2 content[33].

The sheep serum concentrations of 25(OH)D_3 in this study indicate the extent to which vitamin D has been obtained via endogenous photobiosynthesis, since they did not receive any supplementary foodstuffs which contained vitamin D_3, for at least 5 months prior to blood sampling. Lleyn ewes had significantly higher 25(OH)D_3 concentrations, therefore the black heads and legs of the Blackface ewes might have compromised vitamin D_3 photobiosynthesis in those black hair covered areas that are contiguous with dark skin pigmentation[34]. The efficiency of the conversion of 7-Dehydrocholesterol to previtamin D_3 in Scottish Blackface ewes might be compromised because melanin in the pigmented skin competes with 7-Dehydrocholesterol for ultraviolet B photons[35], although the face and limbs might not be the main sites for vitamin D photobiosynthesis in sheep[8]. All ewes were shorn in late June only, so had full fleece cover prior to blood sampling. Breed differences in terms of 25(OH)D_3 could also mean that the Lleyn ewes have a superior capability to retain vitamin D_3 as biologically inert forms such as tachysterol and lumisterol in their body than their BF counterparts[35].

Reports of rickets outbreaks in sheep in Scotland[36,37] indicate that vitamin D deficiency has occurred in geographical locations close to our study site. In regions above latitudes of 55°N, sunlight only provides sufficient intensity of ultraviolet radiation (290–315 nm) to induce a conversion of 7-Dehydrocholesterol to previtamin D_3[38] between mid-March and mid-September[39]. In the Handel *et al.* (2016) study[31], the serum 25(OH)D_3 concentrations in the Soay sheep from St Kilda (57.8°N 8.6°W) were measured in samples taken in August, whereas in this study (56°N 4°W) sheep samples were taken in mid-November. This might explain why the serum 25(OH)D_3 concentrations of sheep in this study were much lower than those of Soay sheep (unadjusted concentration: 21.1 *vs*. 42.8 nmol/l, respectively)[31,39].

Although the vitamin D content in grass is limited, and it is argued that grazing animals obtain vitamin D mainly from endogenous photobiosynthesis[34], our results show that 25(OH)D_2 contributed nearly half of the total 25(OH)D concentration for all three genotypes at the November sampling timepoint. Nevertheless, Lleyn ewes showed significantly higher total 25(OH)D concentrations than Scottish Blackface ewes. This observation of a breed effect was in agreement with Willems *et al.*[40], albeit that different sheep breeds were investigated. The concentration of 25(OH)D has also been reported to be positively associated with skin thickness, which decreases with age[41]. Unlike Handel *et al.*[31], the current study did not find an age-related effect on the vitamin D metabolites investigated, probably due to a narrower age range of ewes (from 1.5 to 6.5 years old at blood sampling time) investigated, whilst Handel *et al.*[31] studied a more age-diverse (from 0.5 to 13 years old) population of feral sheep. The age range considered in this study more or less corresponds with and affirms the 'Adult' category (combining ages from 1 to 6 years) used by Handel *et al.*[31] to distinguish that cohort of Soay ewes from same-breed lambs and geriatric sheep in their study.

In the current study, the breeding outcomes (i.e. number of lambs born or weaned) were not associated with pre-mating ewe status in respect of the vitamin D metabolites investigated. Nevertheless, 9 ewes that lost foetus(es) between scanning and lambing (based on scanning results), had lower concentrations for one or both vitamin D metabolites investigated, compared to the corresponding average unadjusted vitamin D concentrations (Table 2). This indicates that vitamin D might impact on foetal survival in the uterus. Of course, other factors, such as breed differences[42], ewe age[43] or poor nutrition[44], could also contribute and larger studies would be required to fully assess the effect of vitamin D status on foetal survival. In contrast to our findings, the Soay sheep study demonstrated that vitamin D status (August, not November as in our case) was positively associated with annual reproductive success in ewes, i.e. the number of their lambs that survived to one year old[31]. According to the benchmark (vitamin D deficiency: <25 nmol/l, insufficient: 25–75 nmol/l, sufficient: 75–150 nmol/l)

recommended for most species[8], the majority of the ewes in our study were vitamin D insufficient, and in general, their 25(OH)D levels were lower than those reported for Soay sheep in St Kilda, Scotland[31]. However, our sheep were sampled in November, not August, and the general reproductive competence suggests they were not severely compromised[45]. We suggest that these ewes have adapted well to the environmental conditions in north west Scotland, and that their adaptation could include capability to thrive with a vitamin D status below the 'sufficiency' range posited by Nemeth *et al.*[8], at least for part of the year[46,47], as a prerequisite for survival.

Ewes in our study that received the standard feeding level in the first winter feeding period had better body condition at that stage, compared to those with poorer body condition that received the corrective feeding level. These ewes with better condition scores in winter then went on to have greater litter sizes at birth and weaning in the subsequent spring and summer, respectively than their counterparts with relatively poorer body conditions. This is plausible and not unexpected as ewes with good body condition would have increased ovulation rates, reduced risk of embryo loss[48,49], and would produce lambs with heavier birth weights[50,51].

In this study, ewes with higher serum $25(OH)D_3$ and total 25(OH)D concentrations produced heavier lambs for both singleton and twin litter categories. These findings were in line with previous reports of vitamin D status effects in pregnant women[19,22,52,53]. One possible explanation is that high 25(OH)D status could promote local metabolism from 25(OH)D to the active form of vitamin D, $1\alpha,25(OH)_2D$, in the placenta, which supports placental development in early pregnancy[54], and in turn enhances placental transportation of calcium for foetal growth[55,56]. However, this correlation (improved vitamin D status associated with lower incidence of low infant birthweight) has been a controversial issue in human studies, as several authors report different results[57–59]. Environmental conditions are more difficult to standardise in human studies, or those involving wild or feral animals, compared to those conducted on livestock within a research farm environment, which could account for differences in findings across trials and species. Our results suggest that ewes having higher pre-mating vitamin D status might, if there is a pay-off in terms of foetal growth, be favoured in a hill environment, where lamb birth weight is an important determinant of lamb survival in harsh conditions[60,61]. Equally, our results suggest that aforementioned 'sufficient' concentrations[8] may be in excess of what high latitude- and altitude-adapted sheep either could accrue or in fact need to reproduce competently and successfully.

Our results showed that ewes' pre-mating vitamin D status was associated with the lamb birth weight, but not the weight during early growth (marking at around 8 weeks old) or at weaning (around 18 weeks of age). In the early postnatal stage, especially between birth and marking, lamb growth relies mainly on the mother's milk production[62], which apparently was not influenced by pre-mating vitamin D status of ewes in the current study.

Conclusion

Our findings show that the $25(OH)D_3$ and total 25(OH)D concentrations in Lleyn ewes were significantly higher than those in both unimproved and improved Scottish Blackface ewes kept at a Scottish commercial hill farm. The vitamin D status in the 3 genotypes did not have any effects on the number of lambs born or weaned per ewe. The concentrations of $25(OH)D_3$ and 25(OH)D were positively associated with litter weight for singleton and twin lambs at birth, but not at marking or weaning. The relationships between vitamin D status and reproductive outcomes in sheep are deserving of further study.

Data Availability

Data supporting the findings of the current study are available from the corresponding author on reasonable request.

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Author Contributions

P.Z., T.G.M., A.C.G., N.R.L., C.R.M.-D., N.D.S. and R.J.M. conceived and designed the study. P.Z., T.G.M., A.C.G., N.R.L., C.R.M.-D., E.H., N.D.S. and R.J.M. performed the experiments. P.Z., T.G.M., N.R.L., C.R.M.-D., N.D.S. and R.J.M. analysed the data. P.Z., T.G.M., A.C.G., N.R.L., C.R.M.-D., N.D.S. and R.J.M. wrote and reviewed the paper.

Additional Information

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5.3 Conclusion

This is the first publication investigating the effects of vitamin D status on ewe reproductive performance of commercially farmed sheep, specifically ewes of breeding age. The study identified that the concentration of the cutaneously-derived $25(OH)D_3$, but not of the orally consumed $25(OH)D_2$, was significantly higher in Lleyn ewes (these have white hair and fleece and non-pigmented skin) than in their counterpart BF ewes (having white fleece and mostly black skin and hair on their face and legs). The concentrations of $25(OH)D_3$ (and of total 25(OH)D) were positively associated with litter birth weights of singletons and twins. These outcomes in a managed sheep flock were consistent with the previous finding of the effect of vitamin D status on ewe reproductive performance in a feral Soay sheep population. This highlights the importance of ewe pre-mating vitamin D concentrations as these are associated significantly with ovine reproduction.

Chapter 6: Discussion

6.1 Background and aims

Hill sheep farming plays an important role in the extensive areas of Scotland, but is highly dependent on financial support. In the last 20 years, changes in the subsidy payment system and disease outbreaks (e.g. Foot and Mouth Disease) resulted in a significant reduction in the number of breeding ewes in Scotland (The Scottish Government, 2018a,b). Additionally, traditional hill sheep enterprises tend to suffer from low productivity (Carson et al., 2001a). Therefore, improving productivity of sheep enterprises is important for maintaining sustainability of hill farming systems in Scottish rural areas. This PhD study investigated the performance of Lleyn ewes – a lowland/upland prolific sheep breed - co-grazing with genetically selected Scottish Blackface (IBF) ewes (Conington et al., 2006), and unimproved Scottish Blackface (UBF) ewes, as flockmates in the West Highlands of Scotland. The comparisons focused on the performance of ewes and lambs of the three genetic lines. The investigations were conducted in two phases, characterised by different management systems. In the first phase (Chapter 2), the breeding flock comprised approximately 300 ewes per genetic line; and the ewes were managed under either a conventional (CON), or a precision livestock farming (PLF) system, each having different criteria for winter feeding, worming and culling (Morgan-Davies et al., 2014, 2018). In the second phase (Chapter 3) the breeding flock size was approximately 200 ewes per genetic line, with all the ewes being managed under the PLF management system. In order to align with commercial hill farming in Scotland, in this second phase, the ewes were assigned in a balanced manner to either ‘Hill’ (more extensive) or ‘Park’ (moderately extensive) grazing systems, based on genetic line, ewe age and sire family. Pre-lambing metabolic profiling, colostrum quality testing, post mortem examination, grazing observation, measurement of ewe external pelvic width, and examination of vitamin D status in ewes were performed to investigate the reasons for any reproductive differences among the three genetic lines.

6.2 General discussion

A concern surrounding keeping Lleyn ewes in a hill environment is that, as a lowland/upland sheep breed, their reproductive performance and productivity might be compromised when encountering extreme harsh weather conditions and nutritional stress. Thus Lleyn ewes were initially introduced to hill conditions with the flock managed on semi-improved and improved pastures during key times in the production year, to form the first phase of the study. This was representative of Scottish hill farms with a less extensive management system, and aimed to gradually introduce Lleyns to more extensive hill conditions. The results obtained in this phase (Chapter 2) showed that in such moderately extensive conditions, Lleyn ewes had significantly lower barren rates than their UBF and IBF flockmates. They also achieved significantly higher litter sizes at pregnancy scanning, lambing and weaning than UBF and IBF ewes, with heavier average lamb birth weights, and comparable average weaned lamb weights. The weaned litter weight per ewe lambed among Lleyn ewes was comparable to UBF and IBF ewes.

The overall results of the first study phase indicated that Lleyn ewes performed well in the moderately extensive conditions, and no critical issue was raised in relation to animal health and welfare. Therefore, in the second phase (Chapter 3), ewes were further challenged with half of the flock managed under a more extensive Hill Grazing system, whilst the other half was managed in the moderately extensive Park Grazing system (similar to the system in phase 1). In this study phase, with results averaged across the Hill and Park Grazing systems, Lleyn ewes had comparable barren rates to their UBF and IBF counterparts at pregnancy scanning; this figure was lower than that in Chapter 2. The litter sizes (per ewe mated) at pregnancy scanning, lambing and weaning did not differ among the three genetic lines. Lleyn ewes achieved heavier average lamb birth weights than UBF and IBF ewes, and at weaning, the average lamb weight for Lleyn ewes was heavier than or comparable to those for UBF and IBF ewes. Ultimately, Lleyn ewes weaned significantly heavier litters than UBF and IBF ewes.

The average birth weight (averaged across the Hill and Park Grazing systems, 3.75 kg) of lambs born to Lleyn ewes was lighter than that published for Lleyns farmed in both conventional hill and lowland systems elsewhere (3.89 kg; Ceyhan et al.,

2015). Additionally, in the second phase of the investigation, Lleyn ewes farmed under the Hill Grazing system had a lighter average lamb birth weight than their counterparts farmed under the Park Grazing system. It is plausible, and indeed probable that Lleyn ewes, as a lowland/upland sheep breed, cannot achieve their genetic potential when they are farmed in harsher hill conditions with poor nutritional supply. Compared to traditionally farmed BF ewes (Morgan-Davies et al., 2008a), Lleyn ewes proved that they could perform as well as hardy hill sheep breed in a hill environment, by having more lambs born (0.99 *vs.* 1.27 lambs per traditionally farmed BF *vs.* Lleyn ewe mated) with comparable average lamb birth weight (3.5 *vs.* 3.6 kg for traditionally farmed BF *vs.* Lleyns farmed under the Hill Grazing system), and by having more lambs weaned (0.86 *vs.* 1.13 lambs per traditionally farmed BF *vs.* Lleyn ewe mated) with comparable lamb weaning weight (25.8 *vs.* 25.1 kg for traditionally farmed BF *vs.* Lleyns farmed under the Hill Grazing system). For the performance traits investigated, i.e. litter sizes at pregnancy scanning, lambing and weaning, average lamb birth weight, average litter birth weight, average weaned litter weight, results for Lleyn ewes, averaged across the Hill and Park Grazing systems in the second study phase, were better than those achieved by them in the first study phase when they were farmed in moderately extensive hill conditions. Among the second study phase, barren rate, litter size at scanning, lambing and weaning, average litter birth weight and average litter weaning weight were not significantly affected by the interaction of genetic line and management system. The average lamb birth weight of Lleyns farmed under the Hill Grazing system was lighter than that of their counterparts farmed under the Park Grazing system. However, within the Hill Grazing system, this parameter of Lleyn was heavier than those of UBF and IBF counterparts. Most importantly, average lamb birth weights of the six ewe subgroups (three genetic lines by two management systems) were within the optimal birth weight range for maximising lamb survival (Dwyer et al., 2016). Moreover, the weaning data are economically important for farm profitability. Although the lamb weaning weight of Lleyns farmed under the Hill Grazing system was comparable to those of their UBF and IBF counterparts, with higher litter size weaned per Lleyn ewe that lambed comparing to those of UBF and IBF flockmates (averaged across the Hill and Park Grazing systems, which were not affected by the genetic line x management system), weaned litter weights among Lleyns that lambed were heavier than those among UBF and IBF flockmates (averaged across the Hill and Park Grazing systems, which were not affected by the genetic line x

management system). These outcomes suggested that Lleyn ewes could be a good candidate breed for improving output of hill sheep farming enterprises, as they have adapted to their hill environment and outperformed/equalled the performance of a hardy hill sheep breed. Of course, the superior performance of Lleyn ewes in the second study phase could be attributed to genetic selection (Conington et al., 2006; Lambe et al., 2014), reduction of flock size, good ewe body condition (Gunn et al., 1969; Donald & Russell, 1970; Gunn & Doney, 1975), relatively good nutritional supply and relatively good weather conditions in the years studied.

The neonatal lamb mortality rates in the Kirkton flock in the 2015, 2016 and 2017 lambing seasons (12%, 10% and 5%, respectively) were lower than that of a previously recorded Scottish BF flock (18%) from the same region (Morgan-Davies et al., 2008a), and were either equal to or lower than the figures (12% to 19%) reported for purebred or crossbred BF lambs (sire breed: BF, Swaledale, North Country Cheviot, Lleyn and Texel) on six hill farms in Northern Ireland (Speijers et al., 2010). One of the reasons was likely to be that the flock was closely managed in the in-bye fields during lambing. High levels of human intervention would reduce risk of lambs dying from exposure to cold and wet conditions or from hunger. In addition, the majority of the lambs had birth weights within the commonly accepted optimal range of 3 to 5 kg for lamb survival (Conington et al., 2015; Dwyer et al., 2016). Lambs with optimal birth weights are also more likely to express good neonatal behaviours (i.e. standing up, udder seeking and sucking) that are crucial for lamb survival (Dwyer et al., 2016). Importantly, lambs born within the optimal range of birth weights possess superior ability for thermoregulation compared to very light lambs when transferring from the warm maternal uterus to cold external conditions, due to having greater reserves of brown adipose tissue (Symonds & Lomax, 1992). Moreover, the colostrum quality of Lleyn twin-bearing ewes, tested using a Brix refractometer, was as good as that of colostrum from UBF and IBF twin-bearing ewes. Brix refractometry is an easy to use on-farm tool, that determines the refractive index of the colostrum samples, which is associated with the protein and immunoglobulin content of sheep colostrum (Harker, 1978), or the protein content of sheep milk (Gelasakis et al., 2018), or the total solid content of cow milk (Moore et al., 2009). The current outcomes indicate that the abilities of Lleyn ewes to secrete colostrum were not compromised by harsher weather conditions and potentially poorer feed supply encountered in the current study compared to lowland/upland

conditions. Such good quality colostrum secreted by Lleyn ewes, in terms of transferring passive immunity and providing energy to neonates was undoubtedly crucial for Lleyn lamb survival in the hill environment (Dwyer, 2008b). It should be noted, however, that this study only examined the colostrum quality of twin-bearing ewes, and those ewes grazed on in-bye fields with relatively good grass quality before lambing, that could underpin colostrum quality (Al-Sabbagh, 2009). Nevertheless, twin-bearing ewes of the three genetic lines were farmed together, thus the comparison of their colostrum quality should be robust.

In this PhD study, 168 lambs were post mortem examined, in the 2015, 2016 and 2017 lambing seasons. The incidence of lambs dying of starvation/hypothermia was likely kept low by good supervision during lambing, and by optimising lamb birth weight by management of ewe nutrition during pregnancy. Hence, dystocia was the most frequent known cause of neonatal lamb death in the Kirkton flock, in the three consecutive lambing seasons in which post mortem examinations were carried out. This might have been contributed to by the effective diameter of the ewes' pelvic canals being disproportional to the size of their lambs (McSporran & Fielden, 1979). External measurements were made of 689 ewes before mating in September 2016; and the study showed that lamb birth weight, but not ewe external pelvic width, was associated with whether the ewe required assistance at lambing. The external pelvic widths measured in the current study may or may not reflect the internal 'birth canal' dimensions which ultimately determine pelvic influence on ease of lamb delivery. Moreover, the angle between rump and pelvis area has an effect on lambing difficulty (Cloete et al., 1998). Therefore, association of the pelvic size and the pelvic angle with the requirement for ewe assistance at lambing should be further investigated, based on accurately measured ewe pelvises. In addition, over the three lambing seasons, compared to UBF lambs, double the number of IBF lambs (22 *vs.* 45) died as a consequence of dystocia. The genetic selection for increased lamb weaning weights in IBF ewes ought to have led to lamb birth weights being progressively increased, due to positive genetic correlations between these traits (McLaren et al., 2012). This could also have had an impact on lambing difficulty. In the current study, the majority of lambs were still within the aforementioned optimal birth weight range, but birth weights should be monitored if genetic selection for lamb weaning weight is likely to further increase it. Overall, the numbers of lambs

that died due to dystocia for the three genetic lines were relatively low, and therefore it is difficult to pinpoint the exact reasons for differences among the genetic lines.

The nutritional status of twin-bearing ewes in late pregnancy was examined by pre-lambing metabolic profiling. The predicted means of BOHB, albumin, urea N, copper and magnesium were within the published reference ranges for all three genetic lines. These outcomes indicate that, in general, twin-bearing ewes in the Kirkton flock were well nourished, with no early pre-partum signs of metabolic disorders. Another key finding was that Lleyn twin-bearing ewes consistently had significantly higher plasma magnesium concentrations than their UBF and IBF counterparts, in late pregnancy, across the three years. Due to the fact that sheep do not have body reserves of magnesium, plasma concentrations reflect the level of magnesium from diet and are, therefore, a proxy for food intake (Sykes, 2007; Dairy Herd Health and Productivity Service, 2014; Robinson, 2018). Moreover, twin-bearing ewes grazed on in-bye fields where grass quality was relatively good, and supplementary feed was readily available. The presumptively better intakes could explain how Lleyn ewes managed to produce heavier litters than their BF flockmates in the hill environment. Pre-lambing metabolic profiling was only performed in twin-bearing ewes in this study. It would be good if these measurements could be conducted in single-bearing ewes that are managed more extensively, as in the typical commercial hill sheep farming system (where the aim is commonly for one good lamb per ewe per year).

A study of Soay sheep revealed that ewe vitamin D status had a positive association with ewe reproductive performance (Handel et al., 2016). Thus an HPLC-MS/MS method was developed to examine the concentrations of $25(OH)D_2$ and $25(OH)D_3$ in sheep serum samples. The evaluation of the method indicated that this HPLC-MS/MS method is a suitable procedure for measuring $25(OH)D_2$ and $25(OH)D_3$ concentrations. The newly derived method was used to determine vitamin D status in sheep serum samples collected from ewes in November 2015 from the Kirkton flock. The assays showed that Lleyn ewes had significantly higher concentrations of $25(OH)D_3$ (a metabolite of cutaneously-synthesised vitamin D) and 25(OH)D (sum of $25(OH)D_2$ and $25(OH)D_3$) than their UBF and IBF counterparts, but no difference in these parameters was found between the UBF and IBF ewes. The concentrations of these two vitamin D metabolites were also found to be positively associated with

lamb birth weights of singleton and twin litters in the following spring. Therefore, ewe pre-mating vitamin D status could be another proxy marker for lamb birth weight. BF ewes have white fleece, with mostly black skin and hair on their faces and legs that could compromise vitamin D_3 photobiosynthesis in those areas (Holick, 1981; Mearns et al., 2008). In contrast to that, Lleyn ewes have white fleece, with non-pigmented skin and white hair on their faces and legs, and so they could more efficiently convert 7-Dehydrocholesterol to previtamin D_3 in their skin when exposed to sunlight containing sufficient ultraviolet B radiation (Scientific Advisory Committee on Nutrition, 2016). This was, perhaps, why Lleyn ewes could achieve heavier lamb birth weights, compared to UBF and IBF ewes. Examination of ewe pre-mating 25(OH)D_2 and 25(OH)D_3 concentrations also provided novel and informative results, and confirmed that ewe vitamin D status influences reproductive performance (Handel et al., 2016). If vitamin D status of ewes could be determined in different seasons for 2 or 3 consecutive years, that would allow investigation of how vitamin D concentrations fluctuate throughout the ewe reproductive cycle and how these vary across years. This would provide a further account of how vitamin D concentrations influence ewe reproductive performance.

During the five consecutive sheep production years, Lleyn ewes either performed better than, or comparable to, their UBF and IBF counterparts. As previously mentioned, the performance of Lleyn ewes in the more extensive Hill Grazing system requires longer-term investigation. Furthermore, the lifetime performance of Lleyn ewes in a harsh hill environment should be investigated to examine cumulative effects on reproductive performance, for example, after a particularly cold and wet winter or after rearing twins or triplets. Moreover, undernutrition during pregnancy might compromise their offsprings' reproductive performance (Robinson et al., 2002; Hoffman et al., 2018), thus the lifetime performance of Lleyn replacements should be investigated with environmental conditions (i.e. temperature, rainfall and days of snow cover) as a factor in the statistical analysis, which would enable exploration of the effects of those parameters on the performance traits. Additionally, there was heavy snowfall with very low temperatures throughout the winter of 2017-18, through to March 2018, but unfortunately, the ewe performance of that year could not be investigated in this study due to the time constraints.

In the current study, Lleyn ewes were managed in a co-grazing system with UBF and IBF ewes. The better performance of Lleyns might have been at the expense of their BF flockmates, because they possibly preferred to graze in the areas where the overall grass quality was better, as suggested in the grazing behaviour study. Therefore, it would also be valuable if the performance of Lleyn ewes managed under a single breed grazing system could be investigated in a hill farming system.

Overall, this PhD study investigated the solutions (genetic selection of hardy hill sheep breed - BF ewes or a suitable breed substitute – Lleyn ewes) for improving the productivity of hill sheep farming enterprises. The results showed that as a lowland/upland sheep breed, Lleyn ewes outperformed/equalled to hardy hill sheep breed, UBF and IBF ewes, in a Scottish hill farm. Averaged across the Hill and Park Grazing systems, Lleyn ewes had comparable litter sizes and produced heavier lambs at lambing, compared to BF flockmates. They also weaned more lambs among ewes that lambed, with average weaned litter weight heavier than UBF and IBF ewes, which is crucial for farm profitability. Thereby, Lleyn ewes could be a suitable candidate for improving productivity of hill sheep farming enterprises. In addition, Lleyn lambs are likely to finish more quickly and generally produce lambs of superior carcass quality than BF (Signet Breeding Services, 2014; Smith, 2019), and these are other important profit-related traits that could influence the decision to switch breed in a hill sheep farm. Therefore, the results from this thesis could be considered alongside lamb performance and carcass quality traits, and other market relevant traits (e.g. ewe longevity, draft/cast ewe value etc.) for considering options for breed substitution in a hill sheep farm.

6.3 Conclusion

This PhD project investigated the performance of BF and Lleyn ewes in a Scottish hill environment across five consecutive production years. Such a long-term study of two breeds on a hill environment is unique. During the study period, Lleyn ewes achieved comparable litter size (per ewe mated) and significantly heavier litter weight at weaning, compared to UBF and IBF ewes. These are economically important traits for maintaining a financially sustainable hill sheep enterprise. In order to achieve the aforementioned outcomes, Lleyn ewes had higher pre-mating $25(OH)D_3$ and 25(OH)D concentrations than their BF counterparts, and these

vitamin D parameters were positively associated with lamb birth weight. Lleyns also apparently consumed more feed than their BF flockmates in late pregnancy, according to their consistently higher pre-lambing magnesium concentrations across three consecutive years. Consequently, they were capable of achieving comparable litter sizes to and produced heavier lambs at lambing than UBF and IBF ewes. Lamb birth weight had a positive association with lamb growth rate between birth and weaning. The overall results suggest that most Lleyn ewes adapted to the harsh environment and performed well in such conditions. Therefore, Lleyn ewes could be an option for improving productivity of a hill sheep farming system. However, the weather conditions during the study period were not extremely harsh. Thus the resilience and performance of Lleyn ewes in the more extensive Hill Grazing system should be investigated further across a longer time period.

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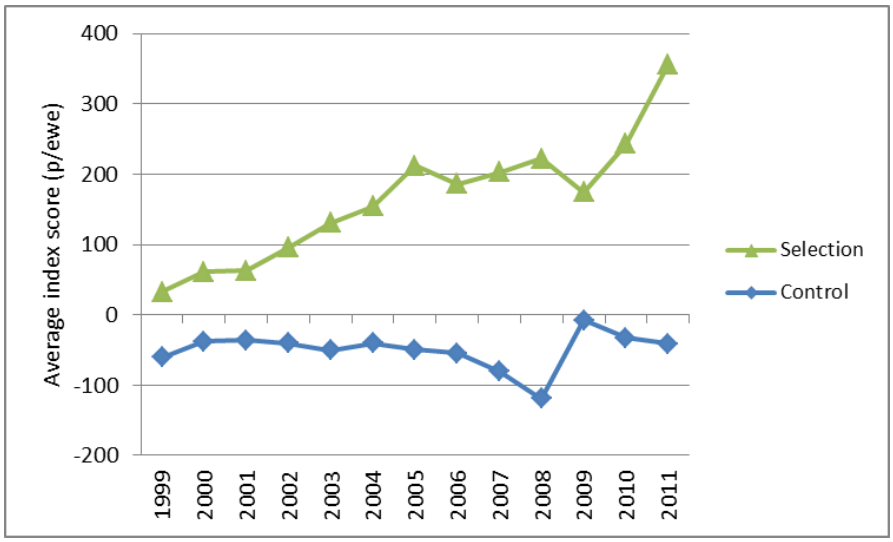
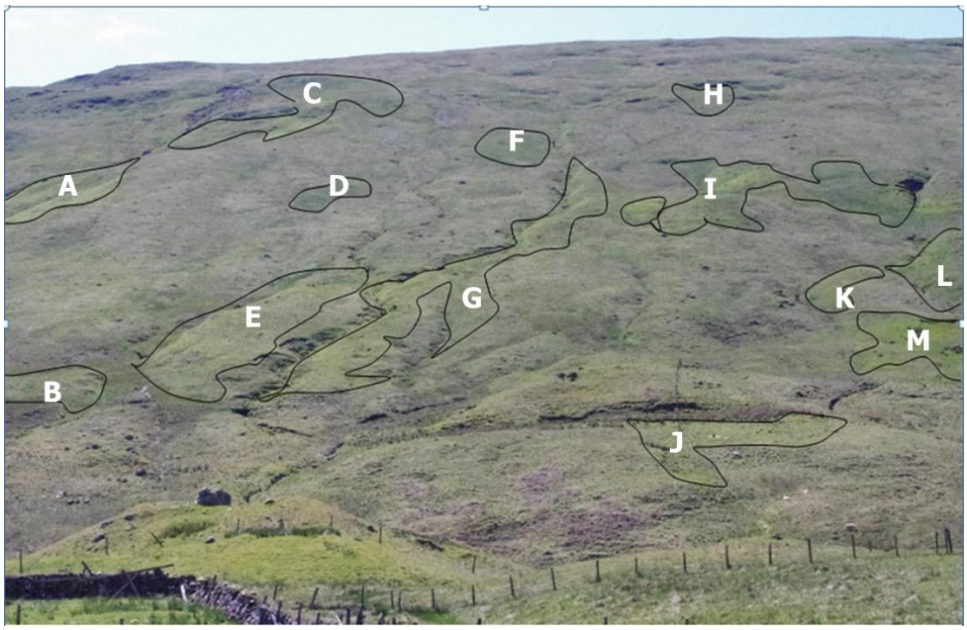
Appendices

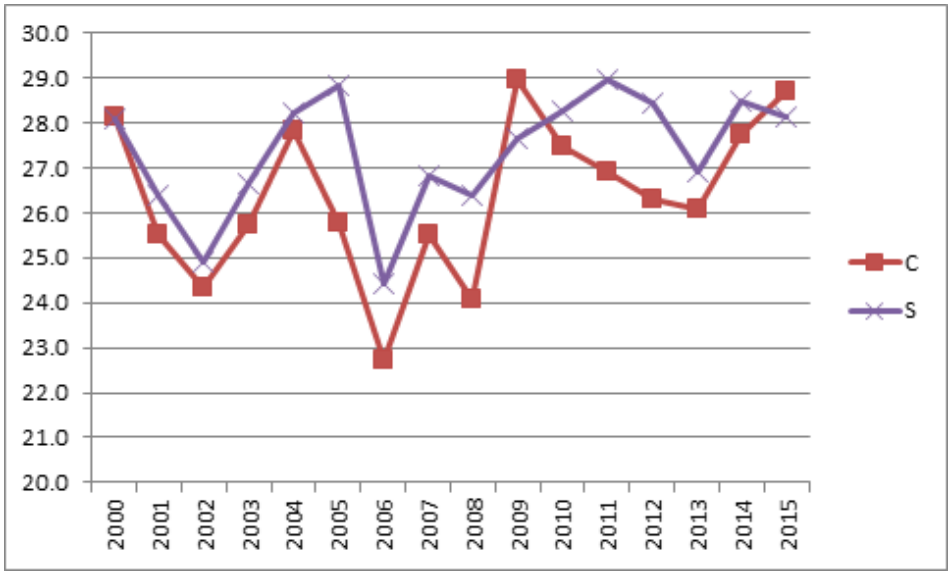
Appendices for Chapter 2



Appendix 2-1. The average economic benefit of the genetic selection (including all the selected traits) over the 13 years (unpublished data adapted from Dr Nicola Lambe). The Control in the figure was equivalent to the UBF ewes, while the Selection in the figure was equivalent to the IBF ewes.

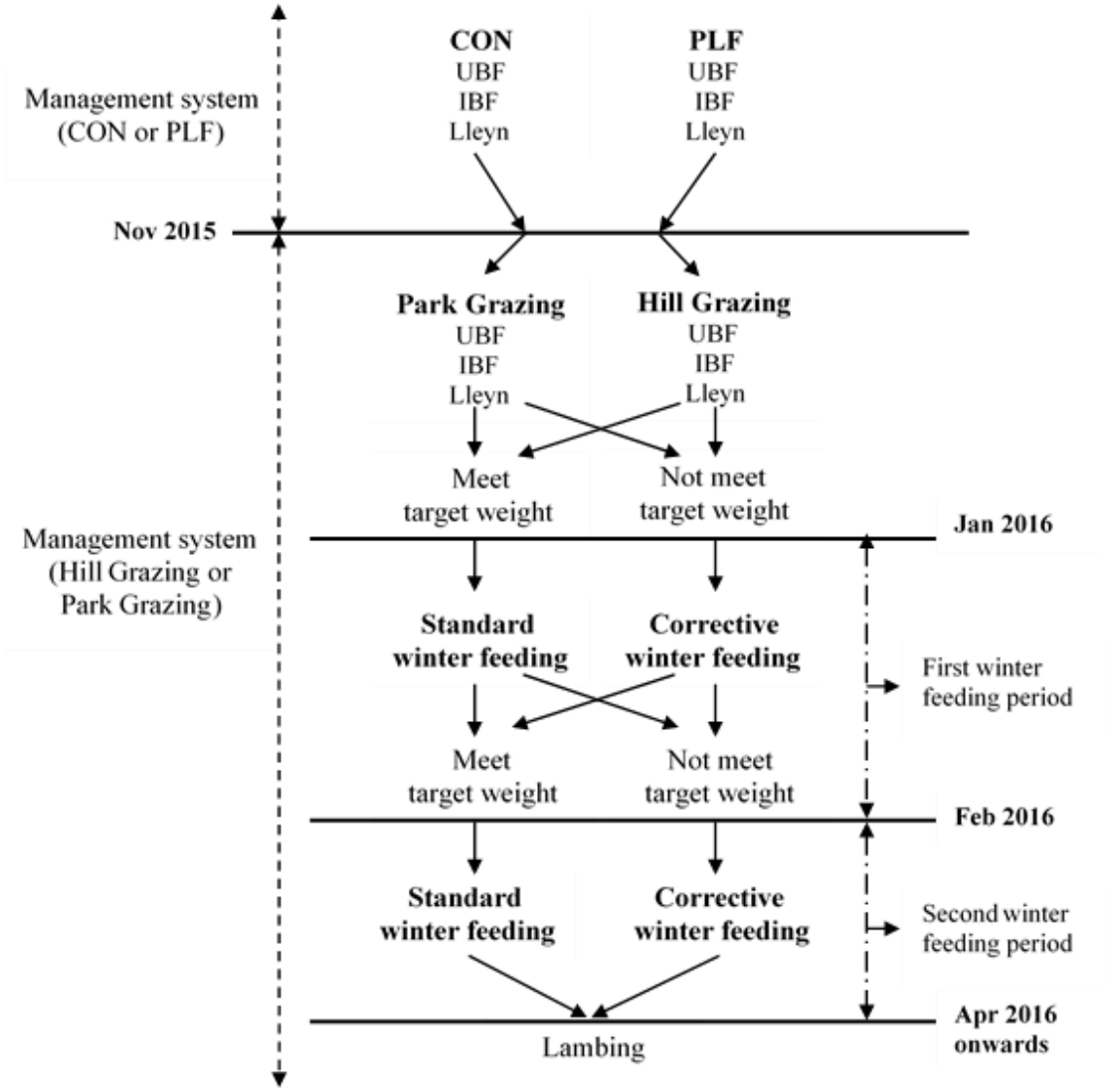


Appendix 2-2. The photograph of the hill site for grazing observation.



Appendix 2-3. The unadjusted lamb weaning weight of UBF and IBF ewes between 2000 and 2015 (unpublished data adapted from Dr Nicola Lambe). The C in the figure was equivalent to the UBF ewes, while the S in the figure was equivalent to the IBF ewes.

Appendices for Chapter 3



Appendix 3-1. Flowchart of management systems and winter supplementary feeding periods in the experiment. The target weight was calculated for every individual ewe.

Appendix 3-2. The total rainfall (mm) of July, August, September, October, November and December in 2012-2016, obtained from Met Office Automatic Weather Station at SRUC Hill & Mountain Research Centre.

Year	July	August	September	October	November	December
2012	129.8	190.8	201.4	172.4	307.2	402.2
2013	94.6	133.4	140.2	281.6	202.8	651.2
2014	108.2	170.6	29.4	491.6	182.4	408.8
2015	249.2	194.0	63.8	173.0	437.2	738.4
2016	170.8	171.2	279.2	82.2	136.6	294.2

Appendix 3-3. The average maximum (max) and the average minimum (min) temperature (°C) of July, August, September, October, November and December in 2012-2016, obtained from Met Office Automatic Weather Station at SRUC Hill & Mountain Research Centre.

	July		August		September		October		November		December	
Year	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**
2012	16.88	9.21	18.53	9.49	14.09	7.10	10.31	1.57	7.77	1.59	5.08	-1.04
2013	20.82	11.42	17.79	10.14	15.16	7.82	12.79	6.58	7.33	-0.51	8.08	2.85
2014	19.83	9.51	16.60	7.94	17.37	8.47	12.64	5.64	9.50	3.23	6.04	-0.36
2015	16.59	8.53	16.79	9.26	16.05	5.65	13.20	3.56	9.05	3.45	8.97	2.50
2016	16.87	9.89	17.57	9.28	16.60	10.09	12.58	4.47	6.46	-1.35	8.94	2.88

Appendices for Chapter 4

Appendix 4-1. Overview of LC-MS/MS methods for quantification of different vitamin D metabolites in biological samples. The mass transitions for each method are listed correspondingly to the order of analyte and internal standard (IS).

Reference	**Analyte and IS**	**Sample preparation**	**LC: i) column ii) mobile phase iii) flow rate**	**MS: i) ionization ii) mass transition**	**Running time (min)**
Aronov et al. (2008)	25(OH)D_2 25(OH)D_3 1α,25-$(OH)_2D_2$ 1α,25-$(OH)_2D_3$ 24R,25$(OH)_2D_3$ IS: d_6-25(OH)D_3 d_6-1α,25$(OH)_2D_3$	500 µl human serum spiked with 20 µl IS at RT for 15 min → protein precipitation (PP; 500 µl acetonitrile) → LLE (500 µl methyl *t*-butyl ether) or offline SPE (Oasis HLB, Waters) → derivatisation (PTAD; 100 ul, 0.75 mg/ml in acetonitrile)	i) BEH C18 (2.1X100 mm, 1.7 µm) at 40˚C; ii) Mobile phase: A = 10% acetonitrile in water v/v & 0.1% formic acid, B = 100% methanol, gradient elution; iii) Flow rate = 0.4 ml/min	i) ESI (electrospray ionization); multiple reaction monitoring (MRM) ii) 570>298, 558>298, 586>314, 574>314, 574>298, IS: 564>298, 580>314	12
Bruce et al. (2013)	25(OH)D_2; 25(OH)D_3;	150 µl of human serum → PP (150 µl of 0.2 M ZnSO4 and 600 µl of IS in	i) Zorbax SB-CN rapid resolution column (100 x	Dinnigan TSQ Quantum Discovery Max triple-	12

Reference	Analyte and IS	Sample preparation	LC: i) column ii) mobile phase iii) flow rate	MS: i) ionization ii) mass transition	Running time (min)
	3-epi-25(OH)D_3; IS: d3-25OH-D_3; d6-3-epi-25OH-D_3	methanol) → SPE (Oasis HLB µelution 96-well plate, Waters), the plate was preconditioned with 200 µl of methanol and 200 µl of ultra-pure water under vacuum, then 750 µl of sample supernatant pass through the well under vaccum. The plate was washed with 200 µl of 5% (v/v) methanol solution, followed by 200 µl of 60% (v/v) methanol solution, then analytes were eluted with 100 µl of methanol into a collection plate with each well containing 34 µl of ultra-pure water.	2.1 mm, 1.8 µm, Agilent); ii) Mobile phase: A = ultra-pure water & 0.1% formic acid, B = methanol & 0.1% formic acid; iii) Flow rate = 350 µl/min	quadrupole mass spectrometer (Thermo Scientific): i) ESI+; SRM; ii) 413.3>395.3; 401.3>383.3; 401.3>383.3; 404.3>386.3; 407.3>389.3	
Geib et al. (2015)	25(OH)D	100 µl of IS and extraction buffer solution (1:2 v/v), and 50 µl human serum → extraction plates (AC Extraction Plate, Tecan, Switzerland)	i) Phenomenex Kinetex PFP (Pentafluorophenyl) 100A column (100 x 2.1 mm, 2.6 µm) at 40°C; ii) Mobile phases A = water + 0.1% formic acid and B = methanol + 0.1% formic acid; iii) Flow rate = 0.4 ml/min	ESI+; MRM; ii) 401>383	10.25
Hedman et al. (2014)	1α,25-$(OH)_2D_2$; 1α,25-$(OH)_2D_3$; IS: d_6-1,25$(OH)_2D_3$; d_6-1,25$(OH)_2D_2$;	200 µl human serum → diluted with 700 µl deionized water → dual column SPE [Chromabond XTR (6 ml, 1 g, Macherey-Nagel) and silica (6 cm^3, 500 mg, Waters) SPE cartridges] → derivatization (Amplifex Diene reagent, AB Sciex)	i) Phenomenex Kinetex (2.4 µm, 3.0 x 150 mm); ii) Mobile phase: A= 0.1% formic acid in 18 MΩ/cm water, B= methanol; iii) Flow rate = 0.25 ml/min	i) Positive TurbolonSpray mode ii) 768.6>689.5 for 1α,25-(OH)2D3-Amplifex Diene	30

Reference	Analyte and IS	Sample preparation	LC: i) column ii) mobile phase iii) flow rate	MS: i) ionization ii) mass transition	Running time (min)
Jenkinson et al. (2016)	25(OH)D_3; 3-epi-25(OH)D_3; 1α,25-$(OH)_2D_3$; 23R,25$(OH)_2D_3$; 24R,25$(OH)_2D_3$; 25(OH)D_2; 24(OH)D_2; 3-epi-25(OH)D_2; 1α,25-$(OH)_2D_2$ 1α,24-$(OH)_2D_2$; Vitamin D_2; Vitamin D_3; 7aC4; IS: 1a,25$(OH)_2D_3$-d_3; 3-epi-25(OH)D_3-d_3; 25(OH)D3-d_3; Vitamin D_2-d_3	220 ul human serum (add 20 µl IS) → PP (80 ul methanol, 50 ul isopropanol and 80 ul water) → Supported liquid-liquid extraction (Phenomenex, Macclesfield, UK) → 2 additions of 800 ul MTBE/ethyl acetate (90:10, v/v) apply to extract the analytes from Supported liquid-liquid extraction wells.	i) Lux Cellulose-3chiral column (2 x 100mm, 3 µm) at 60°C; ii) Mobile phase: methanol/water/0.1% formic acid; iii) Flow rate = 330 µl/min	i) ESI+; MRM; ii)383.2>91.0,383.2>107.0; 383.2>95.4,383.2>107.0; 399.2>105.1, 399.2>151.1; 417.4>325.3, 417.4>343.3; 417.4>121.1, 417.4>381.4; 395.3>91.0, 395.3>119.0; 395.3>340.9, 395.3>119.0; 395.3>91.0, 395.3>119.0; 411.3>133.0, 411.3>151.0; 411.3>133.0, 411.3>151.0; 397.4>69.0, 397.4>107.1; 385.4>107.0, 385.4>259.3; 401.4>97.0, 401.4>117.1; 402.4>138.0, 402.4>154.1; 404.4>107.2, 404.4>109.4; 386.4>95.1, 386.4>109.3; 400.3>109.8, 400.3>69.02	8
Kaufmann et al.(2014)	25(OH)D_2 25(OH)D_3 3-epi-25(OH)D_3 24R,25-$(OH)_2D_3$ IS: d_3-25(OH)D_3 d_6-24,25-$(OH)_2D_3$	100 µl human serum → dilute with 200 µl water → add 100 µl 0.1 M HCL→ PP (0.2 M zine sulphate and 450 µl of methanol) → LLE (700 µl of hexane and 700 µl of methyl tertiary butyl ether) → derivatization (DMEQ-TAD; 2 additions of 25 µl, 0.1 mg/ml in ethyl acetate)	i) BEH-Pheny1 UPLC column (2.1 X 50 mm, 1.7 µm) at 40˚C; ii) Mobile phase: A = 2 mM Ammonium acetate & 0.1% formic acid in water, B = 2 mM ammonium acetate & 0.1% formic acid in methanol; iii) Flow rate = 4 ml/min	i) ESI+; MRM; ii) 758.6>468.3, 746.6>468.3, 746.6>468.3, 762.6>468.3, 749.6>471.3, 768.6>468.3	<5
Kohler et al. (2013)	25(OH)D_2 25(OH)D_3	Sheep and goat serum. Sample preparation was not detailed in the	-	-	-

Reference	Analyte and IS	Sample preparation	LC: i) column ii) mobile phase iii) flow rate	MS: i) ionization ii) mass transition	Running time (min)
		paper			
Maunsell et al. (2005)	25(OH)D_2; 25(OH)D_3; IS: $^2H_6$ 25(OH)D_3	100 µl human serum → PP (75 µl of 60 nmol/L IS in methanol:propanol (80:20)) → LLE (500 µl of hexane)	i) BDS C8 reversed-phase column (51 x 2.1 mm; 3 µm) at 20 ± 0.1°C; ii) Mobile phase: A = methanol and B = 0.5 mL/L formic acid in water; iii) Flow rate = 300 µL/min	i) ESI+; MRM; ii) 413>395; 401>383; 407>389	8
Mena-Bravo et al. (2015)	Vitamin D_2; Vitamin D_3; 25(OH)D_2; 25(OH)D_3; 1α,25-$(OH)_2D_2$; 1α,25-$(OH)_2D_3$; 24R,25$(OH)_2D_3$; IS: 1,25$(OH)_2D_3$–D_6; 24,25$(OH)_2D_3$–D_6; 25(OH)D_3–D_6; Vitamin D_3–D_6; 25$(OH)_2D_2$–D_3; Vitamin D_2–D_3	240 µl human serum spiked with 10 µl deuterated working solution → SPE (Symbiosis system, Spark Hollanad, Emmen, The Netherlands; 10 x 2 mm cartridge packed with Hysphere C8 (Spark Holland), as sorbent material in SPE)	i) Analytical column was Poroshell 120 EC-C18 (2.7 µm, 50×4.6mm, Agilent), while a guard column (2.7 µm, 5.0 × 2.1mm, Agilent) at 15°C; ii) Mobile phase: 5 mM ammonium formate in 85:15 (v/v) methanol–water; iii) Flow rate of 300 µl/min.	Agilent 6410 triple quadrupole mass spectrometer; i) ESI+; selected reaction monitoring; ii) 397.3>107.0, 385.3>107.1, 395.3>133.1, 383.3>159.1, 411.3>133.1, 399.3>147.1, 399.3>121.1	15
Müller et al. (2016)	25(OH)D_2; 25(OH)D_3; 3-epi-25(OH)D_3; 1α,25-$(OH)_2D_3$; 1α,25-$(OH)_2D_2$; 24R,25$(OH)_2D_3$; IS: d_6-25(OH)D_3;	150 µl of IS and extraction buffer (1:2 v/v), and 50 µl of human serum → Supported liquid extraction → Derivatisation (Amplifex Diene Reagent Kit, Canada)	i) Phenomenex Kinetex PFP 100A column (2.6 µm, 100 x 2.1 mm) at 25°C; ii) Mobile phase: A = water & 0.1% formic acid; B = methanol &	i) ESI+; MRM; ii) 744.2>685.4, 732.2>673.5, 732.2>673.5, 748.2>689.5, 760.2>701.4, 748.2>689.5	15

Reference	Analyte and IS	Sample preparation	LC: i) column ii) mobile phase iii) flow rate	MS: i) ionization ii) mass transition	Running time (min)
	d_6-1,25$(OH)_2D_3$; d_6-3-epi-25$(OH)D_3$; d_6-25$(OH)D_2$; d_6-1,25$(OH)_2D_2$; d_6-24,25$(OH)_2D_3$		0.1% formic acid; iii) Flow rate = 0.4 ml/min		
Saenger et al. (2006)	25$(OH)D_2$; 25$(OH)D_3$; IS: $^2H_3$-Δ^9-THC	200 µl human serum + 200 µl IS → LLE (1 ml of heptane) then centrifuge at 3,000 rpm for 4 min → the organic layer was removed, evaporated and reconstituted in 100 µl of ethyl alcohol	i) XTerra analytical column (50 x 2.1 mm, 3.5 µm, Waters) at 35°C; ii) Mobile phase: 2 mmol/l ammonium acetate in methanol with 0.1% formic acid; iii) Flow rate = 100 µl/min	i) ESI+; ii) 413.15>355.2; 401.15>365.25; 318.15>196.20	6
Shah et al. (2011)	25$(OH)D_2$; 25$(OH)D_3$; 3-epi-25$(OH)D_3$; 1αOHD_3; 7αC4; IS: stanozolol-D_3	Unknown volume of serum → PP (2 M formic acid and 3 ml of methanol/isopropanol, 1:1, v/v) → LLE (hexane/dichloromethane, 1:1 v/v) → evaporated and reconstituted with 200 µl methanol/water (1:1, v/v)	i) Agilent microbore ZORBAX SB-C18 RRHD column (2.1 x100 mm, 1.8 µm) was used prior to ULTRON ES-OVM Chiral column (2 x 150 mm, 5 µm) at 40˚C; ii) Mobile phase: A = 0.1% formic acid in acetonitrile and B = 0.1% formic acid in water; iii) Flow rate = 200 µl/min	ESI+; MRM 413.3>377.2; 401.3>383.1; 401.3>365.1; 401.3>365.1; 401.3>365.1; 332.2>81.2	17
Tai et al. (2010)	25$(OH)D_2$; 25$(OH)D_3$;	2 g of human serum spiked with IS (1:1 mass/mass, at RT for 1h) →	i) Zorbax SB CN column (250 x 4.6 mm, 5 µm) at	Applied Biosystmes API 4000 LC-MS/MS:	> 40

Reference	Analyte and IS	Sample preparation	LC: i) column ii) mobile phase iii) flow rate	MS: i) ionization ii) mass transition	Running time (min)
	3-epi-25(OH)D_2; 3-epi-25(OH)D_3; IS: 25(OH)D_2-d_3; 25(OH)D_3-d_3	adjust pH to 9.8 ± 0.2 with carbonate buffer (400 µl at 0.1 g/ml, pH 9.8) → LLE (8 ml of hexane-ehtyl, 50:50, v/v, shaking for 10 min; 2nd LLE with 8 ml of the same solvent shaking for 3 min)	30˚C; ii) Mobile phase: water-methanol (34:66, v/v); iii) Flow rate: 1 ml/min	i) Atmospheric pressure chemical ionization APCI); MRM; ii) 413>395; 401>383; 416>398; 404>386.	
Vogeser et al. (2004)	25(OH)D_3; IS: $^2H_3$,$^{13}C_1$-25(OH)D_3	200 µl serum → addition 30µl of IS (570 nmol/l, equilibrate at 37°C for 2 h) → addition of 20 µl of 1 M NaOH, incubate for 20 min → PP (250 µl of acetonitrile, incubate at 4°C for 1 h) → online SPE (Oasis HLB column, 20 x 2.1 mm, 25 µm bead size, Waters)	i) LiCrospher 100 RP-18 column (125 x 4 mm, 5 µm, Merch); ii), Mobile phase: methanol:0.5 mM ammonium acetate (90:10 v/v); iii) Flow rate = 0.85 ml/min	i) ESI; ii) 401>159; 405>159	9
Yazdanpanah et al. (2013)	25(OH)D_3; 3-epi-25(OH)D_3; 25(OH)D_2; 3-epi-25(OH)D_2; IS: d_6-25(OH)D_2; d_6-25(OH)D_3;	100 µl of human serum spiked with 25 µl of IS solution (d_6-25(OH)D_3 & d_6-25(OH)D_2), dark incubate for 15 min at RT → LLE (1 ml of MTBE) → the ether phase evaporated and reconstituted in 50 µl methanol-water (1:1, v/v) → 20 µl for LC-MS/MS analysis	i) Phenomenex Kinetex PFP column (100 x 3.0 mm, 2.6 µm); ii) Mobile phase: A = water, B = methanol; iii) Flow rate = 800 µl/min	API 4000 QTRAP mass spectrometer: i) APCI+; MRM; ii) 401.2>365.2; 401.2>365.2; 407.2>371.3; 413.2>331.2; 413.2>331.2; 419.2>337.1	7

Appendix 4-2. Overview of serum sample preparations (protein precipitation, liquid-liquid extraction (LLE), solid phase extraction (SPE)) used for determination of vitamin D metabolites.

References	Specimen & analyte	Protein precipitation	LLE	SPE
Adamec et al. (2011)	Serum: Vitamin D_3 with IS: $^2H_6$-D_3; Vitamin D_2 with IS: $^2H_6$-D_2; 25(OH)D_3 with IS: $^2H_6$-25(OH)D_3; 25(OH)D_2 with IS: $^2H_6$-25(OH)D_2	----	Acetone	----
Aronov et al. (2008)	Serum: 25(OH)D_2; 25(OH)D_3; 1α,25-$(OH)_2D_2$; 1α,25-$(OH)_2D_3$; 24R,25$(OH)_2D_3$; IS: d_6-25(OH)D_3; d_6-1α,25$(OH)_2D_3$	500 µl of serum with 500 µl of acetonitrile	500 µl of methyl *t*-butyl ether (MTBE)	Oasis HLB (Waters). Oasis HLB cartridges (3 cc 60 mg) were preconditioned with 3 mL ethyl acetate, 3 mL methanol and 3 mL water. Cartridges were loaded with 900 µL supernatant from the protein precipitation protocol and 1 mL 0.4 M K_2HPO_4. Cartridges were subsequently washed with 3 mL water and 2 mL of 70% methanol and dried for 2 min by application of negative pressure. Samples were eluted with 1.5 mL of acetonitrile into 2 mL plastic tubes.
Baecher et al. (2012)	Serum: 25(OH)D_3; 25(OH)D_2; 24R,25$(OH)_2D_3$; 3-epi-25(OH)D_3; IS: 26,27-D_6-25(OH)D_3	Acetonitrile (300 µl)	----	----
Black et al. (2015)	Serum: 25(OH)D_2; 25(OH)D_3, 3-epi-25(OH)D_3	Methanol-propanol (80:20, v/v)	100 µl of serum sample with 500 µl of hexane	
Bruce et al. (2013)	Serum: 25(OH)D_2; 25(OH)D_3, 3-epi-25(OH)D_3; IS: d_3-25OH-D_3; d_6-3-epi-25OH-D_3	0.2 M ZnSO4 and 600 µl of IS in methanol	----	Oasis HLB µelution 96-well plate from Waters. Each well was preconditioned with 200 µl of methanol, followed by 200 µl of water, then 750 µl of sample supernatants were added. The well was washed with 200 µl of methanol (5%,v/v) and 200 µl of 60% (v/v) methanol. The well was eluted with 100 µl of methanol into a collection plate containing 34 µl of ultra-pure water in each well.

References	Specimen & analyte	Protein precipitation	LLE	SPE
Bunch et al. (2009)	Serum: $25(OH)D_3$; $25(OH)D_2$ IS: $^2H_6$-$25(OH)D_3$	Acetonitrile	----	On-line turboflow extraction (Cyclone-P 50 x 1 mm, Thermo Fisher). Elution solvent was methanol.
Carter et al. (2015)	Serum: $25(OH)D_2$; $25(OH)D_3$; 3-epi-$25(OH)D_3$, $24R,25(OH)_2D_3$	----	[Hexane-ethyl (50:50, v/v) for 2 times] for 25(OH)D and Hexane and MTBE for $24,25(OH)D_3$	----
Chen et al. (2008)	Serum: $25(OH)D_3$; $25(OH)D_2$; IS: $^2H_6$-$25(OH)D_3$	Acetonitrile	----	Online SPE (Oasis HLB cartridge column, 20 x 2.1 mm, 25 µm, Waters). Extraction solution was water-methanol (70:30, v/v), column washing solution was acetonitrile-methanol (50:50, v/v).
Duan et al. (2010)	Serum: $25(OH)D_3$, IS: $^2H_6$-$25(OH)D_3$; $25(OH)D_2$, IS: $^2H_6$-$25(OH)D_2$; $24R,25(OH)_2D_3$, $1\alpha,25-(OH)_2D_3$, IS: $^2H_6$-$1,25(OH)_2D_3$	Methanol-acetonitrile (80:20, v/v)	----	Off-line SPE (Oasis HLB 1-ml, 30 mg sorbent, Waters). The cartridges were conditioned sequentially with 1 ml of acetonitrile for 2 times, and 1 ml of water and 25% acetonitrile with 0.1% formic acid (FA). After loading sample, the cartridge was washed with 1 ml of 30% and 1 ml of 75% acetonitrile (both containing 0.1% FA). Elution solvent was 1 ml of 95% acetonitrile/isopropyl alcohol 4:1 with 0.1% FA.
Farrell et al. (2012)	Serum: $25(OH)D_3$; $25(OH)D_2$; IS: $^2H_6$-$25(OH)D_3$; $^2H_6$-$25(OH)D_2$	$ZnSO_4$ (0.2 M) and methanol	Hexane	Off-line SPE (Oasis uelution HLB plate, Waters).
Geib et al. (2015)	Serum: $25(OH)D_2$; $25(OH)D_3$	Extraction buffer: 0.2M sodium carbonate/sodium hydrogen carbonate 1:1 (v/v) in water/acetonitrile 95:5 (v/v	Extraction plate	----

References	Specimen & analyte	Protein precipitation	LLE	SPE
Gören et al. (2007)	Serum/plasma: $25(OH)D_3$; $25(OH)D_2$; IS: 1a-OHD_3	200 µl of methanol-acetonitrile (70:30, v/v)	Heptane (1000 µl)	----
Hedman et al. (2014)	Serum: $1\alpha,25\text{-}(OH)_2D_2$; $1\alpha,25\text{-}(OH)_2D_3$; IS: d_6-$1,25(OH)_2D_3$; d_6-$1,25(OH)_2D_2$;	Sample was diluted with deionized water, vortex, centrifuge to re-consolidate the liquid	----	Dual column SPE (Chromabond XTR SPE cartridge on the top, 6 ml, 1g, Macherey-Nagel; and silica SPE cartridge, 6 cm^3, 500 mg, Waters). Top cartridge was eluted with di-isopropyl ether (1 ml x 3 min each elution) into the silica column. The silica cartridge was washed with 4% (4.5 ml x 2) isopropyl alcohol in hexanes, followed by 6% (6 ml) isopropyl alcohol in hexanes. Analytes were eluted with 25% (4.5 ml) isopropyl alcohol in hexanes.
Herrmann et al. (2010)	Serum: $25(OH)D_3$; $25(OH)D_2$ IS: $^2H_6$-$25(OH)D_3$; $^2H_6$-$25(OH)D_2$	Acetonitrile (400 µl)	----	----
Hojskov et al. (2010)	Serum: $25(OH)D_3$; $25(OH)D_2$ IS: $^2H_6$-$25(OH)D_3$	Acetonitrile	Automated LLE with heptane	----
Hoofnagle et al. (2010)	Plasma/serum: $25(OH)D_2$; $25(OH)D_3$; IS: Vitamin D_2-d_6; vitamin D_3-d_6	NaOH	n-heptane	----
Jenkinson et al. (2016)	Serum: Vitamin D_2; Vitamin D_3; $25(OH)D_3$; 3-epi-25(OH)D; $1\alpha,25\text{-}(OH)_2D_3$; $23R,25(OH)_2D_3$; $24R,25(OH)_2D_3$; $25(OH)D_2$; $24(OH)D_2$; 3-epi-$25(OH)D_2$; $1\alpha,25\text{-}(OH)_2D_2$; $1\alpha,24\text{-}(OH)_2D_2$; 7aC4; IS: $1\alpha,25(OH)_2D_3$-d_3; $25(OH)D_3$-d_3;3-epi-	Methanol and isopropanol and water	Supportive LLE	----

References	Specimen & analyte	Protein precipitation	LLE	SPE
	25(OH)D_3-d_3; Vitamin D_2-d_3			
Kaufmann et al. (2014)	Serum: 25(OH)D_2; 25(OH)D_3; 3-epi-25(OH)D_3; 24R,25-$(OH)_2D_3$; IS: d_3-25(OH)D_3; d_6-24,25-$(OH)_2D_3$	0.2 M ZnSO4 and methanol	Hexane and MTBE	----
Knox et al. (2009)	Serum: 25(OH)D_3; 25(OH)D_2; IS:$^2H_6$-25(OH)D_3	Methanol	----	ITSP^{TM} SPE (MicroLiter Analytical Supplies, Inc, Suwanne, USA). SPE cartridge was 10 mg Orochem C8. Cartridge was conditioned with methanol and equilibrated with water. Washing with 60% methanol in water (v/v). Elution solvent was methanol. Polarity was adjusted with water.
Kushnir et al. (2010)	Serum: 25(OH)D_3, IS: $^2H_6$-25(OH)D_3; 25(OH)D_2, IS: $^2H_6$-25(OH)D_2	Acetonitrile	----	----
Lensmeyer et al. (2012)	Serum: 25(OH)D_3; 25(OH)D_2; 3-epi-25(OH)D_3	Acetonitrile, water and $ZnSO_4$	----	Off-line SPE (Strata C18 96-well plate, Phenomenex). The sorbent was washed with 1 ml of acetonitrile/water (45:55, v/v). Elution solvent was 1.3 ml of acetone/acetonitrile (20:80, v/v).
Maunsell et al. (2005)	Serum: 25(OH)D_2; 25(OH)D_3 IS: $^2H_6$ 25(OH)D_3	Methanol-propanol (80:20, v/v)	100 µl of serum sample with 500 µl of hexane	----
Mena-Bravo et al. (2015)	Serum: Vitamin D_2; Vitamin D_3; 25(OH)D_2; 25(OH)D_3; 1α,25-$(OH)_2D_2$; 1α,25-$(OH)_2D_3$; 24R,25$(OH)_2D_3$; IS: 1,25$(OH)_2D_3$–D_6; 24,25$(OH)_2D_3$–D_6; 25(OH)D_3–D_6; Vitamin D_3–D_6; 25$(OH)_2D_2$–D_3; Vitamin D_2–D_3	----	----	A 10 x 2 mm cartridge parked with Hysphere C8 (Spark Holland) as sorbent material. Equilibration with 4 ml of 25% acetonitrile with 0.7% formic acid. Washing cartridge with 0.5 ml of 30% acetonitrile. Elution with mobile phase for 5 min (A = 5 mM ammonium formate in methanol, and B = 5 mM ammonium formate in water).

References	Specimen & analyte	Protein precipitation	LLE	SPE
Müller et al. (2016)	Serum: $25(OH)D_2$; $25(OH)D_3$; 3-epi-$25(OH)D_3$; $1\alpha,25\text{-}(OH)_2D_3$; $1\alpha,25\text{-}(OH)_2D_2$; $24R,25(OH)_2D_3$ IS: d_6-$25(OH)D_3$; d_6-$1,25(OH)_2D_3$; d_6-3-epi-$25(OH)D_3$, d_6-$25(OH)D_2$, d_6-$1,25(OH)_2D_2$ and d_6-$24,25(OH)_2D_3$	Extraction buffer: 0.2 M sodium carbonate/sodium hydrogen carbonate 1:1 (v/v) in water/ acetonitrile 95:5 v/v; elution buffer: water/ methanol 10:90 v/v	Supported LLE	----
Priego-Capote et al. (2007)	Serum: Vitamin D_3; Vitamin D_2; $25(OH)D_3$; $25(OH)D_2$; $1\alpha,25\text{-}(OH)_2D_3$	Methanol	*n*-hexane (this is the straight chained structure. hexane has 6 isomers) for 3 times	Sorbent material [500 mg C18 (not endcapped, 14% carbon content, Macherey Nagel, Germany), C18 endcapped (14% carbon content, Macherey Nagel, Germany), and 500 mg Strata C8 (Phenomenex, Torrance, USA). Elution solvent used were methanol, ethanol and isopropanol. the cartridge was activated with methanol and conditioned with acetonitrile. The best results were C8 with isopropanol as elution solvent.
Saenger et al. (2006)	Human serum: $25(OH)D_2$; $25(OH)D_3$; IS: $^2H_3$-Δ^9-THC	----	*n*-heptane	----
Schleicher et al. (2011)	Serum: $25(OH)D_3$; 3-epi-$25(OH)D_3$; IS: d_6-$25(OH)D_3$; $25(OH)D_2$; IS: d_6-$25(OH)D_2$	Methanol (72%, 100 µl)	Hexane	----
Shah et al. (2011)	Serum: $25(OH)D_2$; $25(OH)D_3$; 3-epi-$25(OH)D_3$; $1\alpha OHD_3$; 7αC4 IS: stanozolol-D_3	Formic acid, then methanol-isopropanol (1:1, v/v)	Hexane/dichloromethane (1:1, v/v)	Agilent Technologies Bond Elut-SI silica gel (the best among the sorbent materials used). The cartridges were activated with hexane, then adding methanol, and equilibration with water. Then adding samples. The cartridges were washed with 3 ml water and 3 ml methanol before drying. Elution with 3 ml ether/hexane mixture (30:70, v/v).

References	Specimen & analyte	Protein precipitation	LLE	SPE
Singh et al. (2006)	Serum: $25(OH)D_3$; $25(OH)D_2$; 3-epi-$25(OH)D_3$; 3-epi-$25(OH)D_2$; IS: $^2H_6$-$25(OH)D_3$	200 µl of acetonitrile was added into 200 µl of sample	----	On-line turboflow extraction (Cyclone-P 50 x 1 mm, Cohesive Technologies). After online extraction, the analytes were eluted onto the analytical column for 90 sec with a mobile phase of 39.5% (v/v) methanol, 0.005% (v/v) formic acid.
Stepman et al. (2011)	Serum: $25(OH)D_3$; IS: $^2H_6$-$25(OH)D_3$; $25(OH)D_2$; IS: $^2H_6$-$25(OH)D_2$	NaCl (0.9%, g/g)	n-hexane	----
Strathmann et al. (2012)	Serum: $25(OH)D_3$; 3-epi-$25(OH)D_3$	NaOH	n-heptane	----
Tai et al. (2010)	Serum: $25(OH)D_2$; $25(OH)D_3$; 3-epi-$25(OH)D_2$; 3-epi-$25(OH)D_3$; IS: $25(OH)D_2$-d_3; $25(OH)D_3$-d_3	----	Hexane-ethyl (50:50, v/v) for 2 times	----
Thibeault et al. (2012)	Serum: $25(OH)D_3$, IS: $^2H_6$-$25(OH)D_3$; $25(OH)D_2$, IS: $^2H_6$-$25(OH)D_2$	Acetonitrile using 96-plate filtration system	Hexane	On-line SPE
van den Ouweland et al. (2010)	Human serum: $25(OH)D_3$; $25(OH)D_2$; IS: $^2H_6$-$25(OH)D_3$	NaOH to release protein-bound analyte, and acetonitrile/ methanol (9:1, v/v)	----	Off-line SPE. Strata C18-E columns (55 µm, Phenomenex). Column was pre-equilibrated with 1 ml of methanol then 1 ml of water. Column was washed with 1 ml of water then 1 ml of methanol/water (60:40, v/v). Elution solvent was 250 µl of 100% methanol.
van den Ouweland et al. (2011)	Human serum: $25(OH)D_3$; 3-epi-$25(OH)D_3$; $25(OH)D_2$ IS: $^2H_6$-$25(OH)D_3$	NaOH and acetonitrile-methanol (9:1, v/v)	----	Off-line SPE. Strata C18-E column (55 µm, Phenomenex). Column was pre-equilibrated with methanol then water. Column was washed with water then methanol/water (60:40, v/v). Elution solvent was 100% methanol.
Vogeser et al. (2004)	Serum: $25(OH)D_3$;	NaOH and acetonitrile	----	Oasis HLB column (20 x 2.1 mm, 25 µm). The mobile phase water-methanol (95:5, v/v) at flow rate of 3 ml/min. Extraction

References	Specimen & analyte	Protein precipitation	LLE	SPE
	IS: $^{2}H_{3,}{}^{13}C_{1}$-25(OH)D_3			column was washed with acetonitrile-methanol (50:50, v/v) at flow rate of 3 ml/min for 3.5 min. Reequilibration with water-methanol (95:5, v/v).
Wagner et al. (2011)	Serum: 25(OH)D_3; 25(OH)D_2; 24R,25$(OH)_2D_3$ IS: $^{2}H_6$-25(OH)D_3	----	MTBE then heptane	----
Xie et al. (2011)	Serum: Vitamin D_3; IS: $^{2}H_6$-D_3	----	MTBE extraction (twice)	Solid phase micro-extraction in 96-well format (washing solvent was water with 5% methanol (v/v); eluting solvent was methanol)
Yazdanpanah et al. (2013)	Serum: $25(OH)_3$; 3-epi-25(OH)D_3; 25(OH)D_2; 3-epi-25(OH)D_2; IS: d_6-25(OH)D_2; d_6-25(OH)D_3	----	MTBE	-----

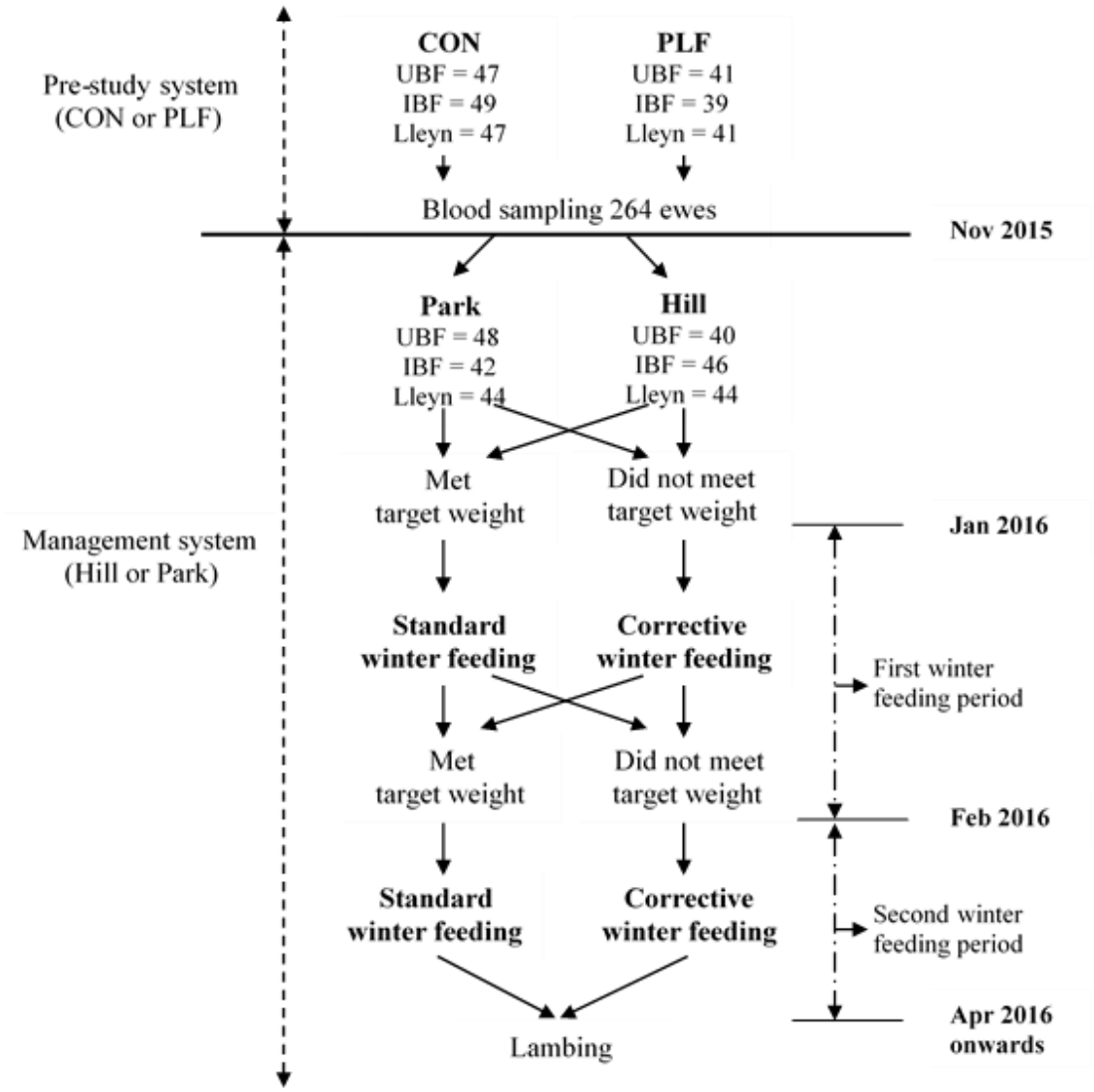
Appendix 4-3. Concentrations of $25(OH)D_2$ (nmol/l) and $25(OH)D_3$ (nmol/l) of 10 Soay sheep serum samples reported by the Supra Regional Assay Service Laboratory and obtained using the method developed in Chapter 4.

	Supra Regional Assay Service Laboratory		**Method developed in Chapter 4**	
	$25(OH)D_2$	**$25(OH)D_3$**	**$25(OH)D_2$**	**$25(OH)D_3$**
Soay sheep 1	10.3	43.8	13.60	40.04
Soay sheep 2	7.91	31.5	13.07	26.14
Soay sheep 3	21.8	35.4	24.98	33.07
Soay sheep 4	9.87	12	9.66	13.42
Soay sheep 5	19.8	52	24.58	68.60
Soay sheep 6	17.6	47.5	29.47	56.83
Soay sheep 7	6.24	16.8	8.58	24.08
Soay sheep 8	9.01	61.5	10.17	43.33
Soay sheep 9	15.2	95.9	15.46	71.64
Soay sheep 10	37.4	38.3	47.92	37.88

Appendices for Chapter 5

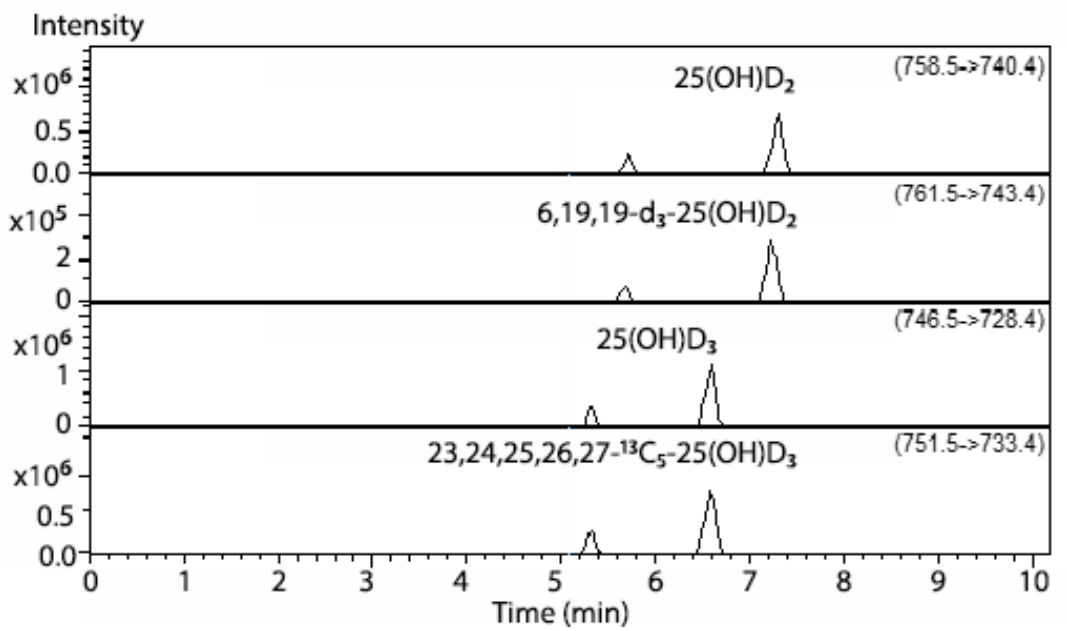
Below are supplementary materials used in the Chapter 5

Supplementary Figure S1.



Supplementary Figure S1. Flowchart of management systems and winter supplementary feeding periods in the experiment. The target weight was calculated for every individual ewe.

Supplementary Figure S2.



Supplementary Figure S2. The chromatogram of a standard solution of 25(OH)D_2 (28.85 nmol/l) and 25(OH)D_3 (44.58 nmol/l). Multiple reaction monitoring was at 758.5>468.1 for 25(OH)D_2, and at 746.5>468.1 for 25(OH)D_3.

Supplementary Table S1.

Protein (%)	18	**Sodium (%)**	2.7
Urea (%)	2.5	**Zinc (mg/kg)**	500
Oil (%)	7	**Manganese (mg/kg)**	250
Fibre (%)	3	**Iodine (mg/kg)**	20
Ash (%)	19	**Cobalt (mg/kg)**	2.5
ME (MJ/kg DM)	13	**Selenium (mg/kg)**	2.5
Calcium (%)	3.3	**Vitamin A (iu/kg)**	25,000
Phosphorus (%)	0.27	**Vitamin D_3 (iu/kg)**	5,000
Magnesium (%)	2	**Vitamin E (iu/kg)**	500

Supplementary Table S1. Product specification of Rumevite Sheep Super Energy plus Fish Oil. Rumenco Rumevite Sheep Super Energy plus Fish Oil mineral blocks were supplied during winter supplementary feeding periods in 2016.

Supplementary Table S2.

	Ewe age at sampling (years)					
	1.5	**2.5**	**3.5**	**4.5**	**5.5+**	**Total**
UBF	15	43	11	9	10	88
IBF	15	39	15	6	13	88
Lleyn	19	33	19	2	5	88

Supplementary Table S2. The distribution of ewes by age at blood sampling for the 3 genotypes.

Supplementary Table S3.

LC parameters	
Injection volume	5 µl
Flow rate	200 µl/min
Column temperature	40 °C
Injection needle rinse	20% Methanol
Mobile phase gradient:	
0 min	Sample injection
0 min to 1 min	20% B to 72% B
1 min to 7.5 min	72% B
7.5 min to 9 min	72% B to 100% B
9 to 9.1 min	100% B to 20% B
9.1 to 10 min	20% B
Equilibration	2 min of 20% B
Total run time	12 min
MS parameters	
Electrospray ionisation mode	Positive
Capillary current	4500 V
Nebulizer gas	Nitrogen; 16 psi
Dry gas flow rate	8.0 L/min
Dry gas temperature	220°C

Supplementary Table S3: Instrumentation set-up for HPLC-MS/MS analysis. The top panel was for liquid chromatography, while the bottom panel was for mass spectrometry.

Supplementary Table S4.

Analyte	Mass transition	Fragmentation cut-off *m/z* and fragmentation amplitude, *v*	Retention time (min)
25(OH)D_2	758.5>740.4>468.1	1st precursor: 163/0.90; 2nd precursor: 167/0.53	5.7 & 7.3
6,19,19-d_3-25(OH)D_2	761.5>743.4>471.1	1st precursor: 184/0.95; 2nd precursor: 167/0.53	5.7 & 7.3
25(OH)D_3	746.5>728.4>468.1	1st precursor: 185/0.70; 2nd precursor: 164/0.80	5.4 & 6.6
23,24,25,26,27-$^{13}C_5$-25(OH)D_3	751.5>733.4>468.1	1st precursor: 185/0.70; 2nd precursor: 164/0.80	5.4 & 6.6

Supplementary Table S4: The mass transitions and the optimised conditions for the selected MS/MS method for detecting 25(OH)D_2 and 25(OH)D_3 as well as their corresponding internal standards (i.e. 6,19,19-d_3-25(OH)D_2 and 23,24,25,26,27-$^{13}C_5$-25(OH)D_3, respectively).

The major characteristic ions for 25(OH)D_2 and 25(OH)D_3 derivatives were at a mass/charge (m/z) ratio of 758.5 and 746.5, respectively. The optimised conditions (fragmentation cut-off and amplitude) for these 2 vitamin D metabolites were detected using a direct infusion method with a syringe pump. Under optimised HPLC-MS/MS conditions, each of 25(OH)D_2 and 25(OH)D_3 derivatives generated 2 main fragments, which were a DMEQ-TAD fragment with m/z ratio of 247.0 and A-ring/DMEQ-TAD with m/z ratio of 468.1. Both fragment ions were used in the multiple reaction monitoring during tandem mass spectrometry. Two isomers, 6R and 6S were produced during derivatisation, thus there were 2 peaks for each analyte in the resultant chromatogram.

Supplementary Table S5.

Analyte	r^2	LLOD (nmol/l)	LLOQ (nmol/l)	Calibration range (nmol/l)
25(OH)D_2	0.998	3.6	7.2	1.8 – 230.8
25(OH)D_3	0.996	5.6	5.6	2.8 – 356.6

Supplementary Table S5: Details of calibration curves.

The derivative of 25(OH)D with DMEQ-TAD reagent comprised 6S and 6R isomers. The major product, 6S isomer generated a larger peak, which was used for quantifying the quantity of the analyte. The calibration curve was constructed by using the ratio of the larger peak area of the standard to that of the corresponding internal standard (i.e. 25(OH)D_2-d_3 or $^{13}C_5$-25(OH)D_3). The correlation coefficients (r^2) were calculated from the means of 8 calibration curves. Lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were determined by identifying the lowest standard contained within the standard curve that had a minimum signal to noise ratios of 5:1 and 10:1, respectively, and for which observed concentration was within 30% of intent.

Supplementary Table S6.

Analyte	Injection carryover (%)	Recovery rate (%)	Intra assay coefficient variation (%)	Inter assay coefficient variation (%)
25(OH)D_2	0.01	63	7.5	17.3
25(OH)D_3	0.02	54	6.9	15.1

Supplementary Table S6: Method validation of the HPLC-MS/MS analysis of 25(OH)D_2 and 25(OH)D_3 in sheep serum samples.

Injection carryover was determined by comparing the peak areas of the analytes in the validation run of the highest concentration standard with the corresponding areas in the bland [60:40 (vol:vol) methanol with 0.1% formic acid:water], that was analysed immediately afterwards. The injection carryovers reported here were the means of 8 runs. The recovery rate of sample preparation was examined by comparison of the artificial serum spiked with stock standards (and then put through the sample preparation procedures demonstrated in the method) with the post-extracted samples. The post-extracted samples were generated by spiking the same amount of stock standards into a tube that contained dried artificial serum which had already gone protein precipitation and solid phase extraction. Three concentrations [233.0, 116.5 and 58.3 nmol/l for 25(OH)D_2; 234.0, 120.0 and 60.0 nmol/l for 25(OH)D_3] of samples were prepared in duplicates with internal standards. The total peak areas of 6S and 6R isomers were used for calculating the recovery rate. Each sample or calibration standard was analysed in duplicate throughout HPLC-MS/MS analysis. Intra-assay coefficient variation was obtained from 5 runs (consisting of the results of 88 sheep serum samples). Inter-assay coefficient variation was determined on the basis of results from four Scottish Blackface sheep serum samples analysed in 3 consecutive runs.

Supplementary Table S7. Summary of fixed models and random models used in the LMM statistical analyses.

Response variate category	Response variate	Final fixed model	Random model
Vitamin D status	25(OH)D_2 concentration	Ewe breed/genotype + ewe age + ewe pre-mating weight	Batch
	25(OH)D_3 concentration	Ewe breed/genotype + ewe pre-mating weight + number of lambs weaned in the last breeding cycle + weaned litter weight in the last breeding cycle	Batch
	25(OH)D concentration	Ewe breed/genotype + ewe pre-mating weight + number of lambs weaned in the last breeding cycle + weaned litter weight in the last breeding cycle	Batch
Ewe breeding outcomes	Number of lambs born	25(OH)D_2/25(OH)D_3/25(OH)D concentration + ewe genotype + ewe age + management system + pre-study system + ewe pre-mating weight + number of lambs weaned in the last breeding cycle + first winter feeding	Batch + ram group
	Number of lambs weaned	25(OH)D_2 concentration + ewe genotype + ewe age + management system + pre-study system + ewe pre-mating weight + ewe pre-mating CS + number of lambs weaned in the last breeding cycle + first winter feeding	Batch + ram group
		25(OH)D_3/25(OH)D concentration + ewe genotype + management system + pre-study system + ewe pre-mating weight + ewe pre-mating CS + number of lambs weaned in the last breeding cycle + first winter feeding	Batch + ram group
Ewe litter weights to weaning (dam dependent)	Singleton birth weight	25(OH)D_2 concentration + ewe genotype + management system + first winter feeding + ewe pre-mating weight	Batch + ram group
		25(OH)D_3/25(OH)D concentration + ewe genotype + management system + first winter feeding	Batch + ram group
	Singleton marking weight	25(OH)D_2/25(OH)D_3/25(OH)D concentration + ewe genotype + management system + first winter feeding + ewe pre-mating weight	Batch + ram group
	Singleton weaning weight	25(OH)D_2/25(OH)D_3/25(OH)D concentration + ewe genotype + first winter feeding + ewe pre-mating weight	Batch + ram group
	Twin litter birth weight	25(OH)D_2 concentration + ewe genotype + ewe age + pre-study system + ewe pre-mating weight + ewe pre-mating CS	Batch + ram group
		25(OH)D_3/25(OH)D concentration + ewe genotype + management system + ewe pre-mating weight + ewe pre-mating CS	Batch + ram group
	Twin litter marking weight	25(OH)D_2/25(OH)D_3/25(OH)D concentration + ewe genotype + management system + first winter feeding + ewe pre-mating weight + ewe pre-mating CS	Batch + ram group
	Twin litter weaning weight	25(OH)D_2/25(OH)D_3/25(OH)D concentration + ewe genotype + management system + first winter feeding + ewe pre-mating weight + ewe pre-mating CS	Batch + ram group